# CXXVII. DETERMINATION OF IODINE IN SHEEP THYROID

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In a recent paper [Sigurjonsson, 1938] I showed that in Iceland the human thyroid gland, being unusually small, is relatively very rich in iodine, containing on the average 0·834 mg. iodine per g. of fresh substance. This indicates a very high iodine intake. Now, whilst in regions where goitre is common and the "normal" thyroid is relatively large, the soil and water usually show low iodine contents, one cannot conversely deduce an exceptionally high iodine content of the soil from the prevalence of small and iodine-rich human thyroids, since the human being is not entirely dependent on food which derives its iodine from the soil. On the other hand, the thyroids of herbivorous animals such as the sheep, may serve as a fairly good indicator of the iodine content of the soil and water where they are grazed.

In this paper are reported the iodine contents of 33 sheep thyroids collected late in October 1937 at the slaughterhouse in Reykjavik. The sheep came from different districts of the south-western part of Iceland, where, during the summer months, they were grazed on the high areas.

The thyroid glands were carefully dissected from the adjacent connective tissue and fat and then weighed. A small piece was cut out and weighed for histological examination, and the rest minced, dried and pulverized. Then an aliquot, usually 0·2 g. of the dry powder, was taken for analysis.

One of the most delicate steps of the determination of iodine in organic matter is the ashing process. Even in the presence of sufficient alkali, the heat requires careful attention if no iodine is to be lost by volatilization. Ashing in an electric oven, where the temperature can be easily controlled, is therefore greatly to be preferred. Further, in wet ashing, frothing and sputtering may cause some trouble; I therefore used a pulverized mixture of $Na_2CO_3$, $K_2CO_3$ and $KNO_3$, recommended to me by the Zoophysiological Laboratory in Copenhagen [see also Kraus, 1915]. Thereafter I proceeded on much the same lines as in the method of Kendall as modified by Kelly & Husband [1924].

*Reagents required:*

Ashing powder: $Na_2CO_3$, anhydrous, 353 g.; $K_2CO_3$, anhydrous, 460 g.; $KNO_3$, 250 g.
$H_2SO_4$: 33 % (by volume).
Methyl orange.
$NaHSO_3$: 20 %.
Bromine.
$Na_2SO_3$: $N/100$.
KI.
Soluble starch: 1 %.
$KIO_3$: standard solution.

*Outline of method.* 0·2 g. of the powdered thyroid is mixed with 10 g. of the ashing powder in a nickel crucible, and covered with 5 g. of the powder. The crucible with cover is then placed in an electric oven and heated. As the ashing proceeds brownish fumes rise through the covering layer of the powder, which itself becomes dark brown. Towards the end of the ashing process, the brown colour completely disappears, and the white mass tends to retract from the

walls of the crucible. The $KNO_3$ greatly facilitates the ashing, which is completed in about $1\frac{1}{4}$ hr., at a final temperature about 400° (never exceeding 450°).

The crucible is then placed in a beaker with 100 ml. boiling water, and the melt dissolved. The solution which is perfectly clear and colourless, is transferred to an Erlenmeyer flask, the crucible with cover and the beaker are washed with distilled water, which is added to the solution and the volume made up to *c.* 400 ml. with distilled water. It is then neutralized with $H_2SO_4$ (methyl orange) and a few drops excess acid added. The liberated $HNO_2$ is eliminated by boiling 15–20 min. (controlled by KI-starch paper), 1 ml. $NaHSO_3$ is added and boiling continued 10 min. to expel $SO_2$. Then freshly made bromine water is added to a distinct brown colour and the excess of bromine expelled by boiling *c.* 10 min. (controlled with KI-starch paper). Then after cooling 3 ml. of 10% KI are added and the liberated iodine titrated with $N/100$ $Na_2SO_3$.

The ashing powder and other reagents must be tested for iodine content by a blank analysis.

In Table I are illustrated some results showing the accuracy of the method.

Table I

| Amount of dry thyroid, No. 13 g. | Iodine added as KI mg. | Iodine recovered mg. | Error in % |
|---|---|---|---|
| — | 0·100 | 0·108 | +8·0 |
| — | 0·100 | 0·099 | −1·0 |
| — | 0·200 | 0·193 | −3·5 |
| — | 0·300 | 0·311 | +3·7 |
| — | 0·400 | 0·394 | −1·5 |
| — | 0·500 | 0·508 | +1·6 |
| 0·2 | — | 0·595 | — |
| 0·2 | — | 0·616 | — |
| 0·2 | — | 0·618 | — |

Table II shows the iodine contents of the thyroids of lambs about 5 months old, and Table III that of the thyroids of sheep 17 months and older.

Table II*

| Weight of thyroid in g. | I content in mg. per g. of | | Total I content of thyroid |
|---|---|---|---|
| | Fresh weight | Dry weight | |
| 1·85 | 0·945 | 3·365 | 1·748 |
| 1·97 | 0·844 | 2·963 | 1·663 |
| 1·62 | 1·058 | 3·545 | 1·714 |
| 1·37 | 1·421 | 4·847 | 1·947 |
| 1·17 | 1·112 | 3·693 | 1·302 |
| 1·87 | 1·061 | 3·598 | 1·984 |
| 1·23 | 0·988 | 3·990 | 1·215 |
| 1·75 | 0·963 | 3·672 | 1·685 |
| 1·58 | 1·138 | 3·746 | 1·798 |
| 1·54 | 1·038 | 3·323 | 1·598 |
| 1·21 | 1·072 | 3·418 | 1·296 |
| 1·58 | 1·725 | 5·926 | 2·625 |
| 1·40 | 0·960 | 3·524 | 1·344 |
| 1·42 | 0·990 | 3·820 | 1·406 |
| 1·40 | 1·519 | 4·709 | 2·126 |
| 1·88 | 1·222 | 4·423 | 2·297 |
| Average 1·55 | 1·128 | 3·910 | 1·734 |

* It is to be noted that the figures of the fresh weight may be somewhat too low, as the glands were not collected until several hours after slaughtering, and may therefore have lost some weight during that time. This again will account for too high findings for the iodine content of the fresh substance, whereas the iodine content of the dry substance is not affected.

The histological picture of the lamb thyroids was fairly uniform, the acini small, 50–150 $\mu$ in diameter and smaller. In the thyroids of the older sheep, the acini showed more variation in size, and were on the whole larger, 100–300 $\mu$ being the predominating size, and some glands had a distinctly colloid character. In no case were there any signs of hyperplasia to be seen.

Table III*

| | Weight of thyroid in g. | I content in mg. per g. of | | Total I content of thyroid in mg. |
|---|---|---|---|---|
| | | Fresh weight | Dry weight | |
| | 3·07 | 1·543 | 5·291 | 4·737 |
| | 3·07 | 1·334 | 4·815 | 4·095 |
| | 1·62 | 1·224 | 4·466 | 1·983 |
| | 3·35 | 0·878 | 3·048 | 2·941 |
| | 2·15 | 1·179 | 3·831 | 2·534 |
| | 2·33 | 1·274 | 4·127 | 2·968 |
| | 2·82 | 0·902 | 3·175 | 2·541 |
| | 3·37 | 0·932 | 3·143 | 3·143 |
| | 2·62 | 1·856 | 6·032 | 4·862 |
| | 3·30 | 1·354 | 4·360 | 4·468 |
| | 2·30 | 0·714 | 2·646 | 1·642 |
| | 3·00 | 1·160 | 3·513 | 3·480 |
| | 4·60 | 0·731 | 2·392 | 3·363 |
| | 3·90 | 1·576 | 4·762 | 6·146 |
| | 3·45 | 1·186 | 3·820 | 4·092 |
| | 4·40 | 1·091 | 3·492 | 4·800 |
| | 3·80 | 1·629 | 5·476 | 6·190 |
| Average | 3·13 | 1·210 | 4·023 | 3·764 |

* See note to Table II.

## Discussion and conclusions

The number of thyroids here examined is indeed too small for far-reaching conclusions to be drawn, but as the results are fairly uniform and as the sizes of a great number of glands observed at the same time were apparently of the same order, I believe that the results will not differ much from the truth.

From Tables II and III it is seen that the relative iodine content of the lamb thyroid is practically the same as of the thyroid of the older sheep, and the average for the two groups taken together is 3·968 mg. per g. of dry substance, or 0·397 %. This is very similar to the results of Martin [1912; 1913], who on two occasions found respectively 0·343 and 0·407 % of iodine in the thyroids of sheep from the neighbourhood of Newcastle. This may be taken as an indication that the pastures in the two localities have iodine contents of somewhat the same order. In the tables of Martin no distinction is made between the thyroids of lambs and of older sheep, and therefore the sizes are not directly comparable, although it seems that here also there is a close relationship, as was to be expected from the relative iodine contents.

Marine & Lenhart [1909] found the iodine content of non-goitrous sheep thyroids in Ohio to be only 0·247 % of the dry substance. In this district goitre was not infrequent, and the normal sheep thyroid was relatively large, the 19 glands examined ranging from 4·5 to 8·5 g. in weight. Also most other authors from different localities, as quoted by Orr & Leitch [1929], have found lower values for iodine content. It therefore seems that the pastures in the above-mentioned districts of Iceland and North England are among the richer in iodine.

While the iodine content of the soil in this particular district in Iceland may thus be assumed to be adequate to prevent endemic goitre in the human population, it does not seem to be high enough to be responsible alone for the above-mentioned smallness of the human thyroid, since the vegetables cultivated in this country only constitute a minor part of the food.

Other foodstuffs, particularly the iodine-rich fish, undoubtedly play a great role here. Further investigations are to be made on this point.

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# CXXVIII. METABOLISM OF THE STRICT ANAEROBES (GENUS: *CLOSTRIDIUM*)

## II. REDUCTION OF AMINO-ACIDS WITH GASEOUS HYDROGEN BY SUSPENSIONS OF *CL. SPOROGENES*

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Studies were made of the conditions under which suspensions of *Cl. sporogenes* activate certain substrates as H-acceptors. Gaseous $H_2$ was used as the H-donator and the reactions carried out in Warburg manometers, in contrast to the methods used by other workers [Stickland, 1934; Woods, 1936]. It is believed that some of the objections to previous methods are eliminated by the method used in these studies. The method became possible only after it was found that *Cl. sporogenes*, under the proper conditions, actively utilizes gaseous $H_2$.

### Experimental

The strain of *Cl. sporogenes* used in these experiments was obtained from the American Type Culture Collection and was labelled No. 319. The organisms were cultured at *p*H 7·3 in 3% neopeptone solution, to which 1% glucose was added. Anaerobic conditions were provided in McIntosh and Fildes jars. Following incubation for 16 hr. at 37° the culture was centrifuged. The precipitated organisms were then washed with a volume equal to the original culture of 0·85% NaCl which had been previously boiled and cooled. After recentrifuging the organisms were resuspended in a previously boiled and cooled phosphate buffer solution of *p*H 7·2. Usually the organisms obtained from 250 ml. culture were suspended in 6 ml. phosphate buffer.

One ml. suspension of *Cl. sporogenes* in phosphate buffer was placed in each Warburg manometer vessel, the side arm of which contained 0·5 ml. 0·1*M* solution of the prospective H-acceptor. $H_2$, purified by passing through a column of electrically heated copper particles, was directed through the apparatus for 10 min., after which the vessels were closed and placed in a water bath at 40°. After equilibrium had been reached (*c.* 5 min.) the substrate was added from the side arm and the change in gaseous pressure (in this case a decrease) measured. A control experiment in which the bacterial suspension and the substrate solution were not mixed together was carried out simultaneously.

In the first experiment glycine and hydroxyproline were used. Stickland [1934], employing another technique, had shown that these compounds acted as H-acceptors. As may be seen from Fig. 1 a very marked and uniform $H_2$ absorption with no decrease in rate even after 3–4 hr. was observed when either of these

compounds was added to the suspension of *Cl. sporogenes*. With glycine the absorption was slightly greater than with hydroxyproline. There was no appreciable $H_2$ absorption in the control.

The presence of glucose in the culture medium, while not entirely essential, was found to increase very markedly the ability of *Cl. sporogenes* to activate gaseous $H_2$. In the absence of glucose some $H_2$ was usually absorbed but the amount was very small, a heavy bacterial suspension being required in order to detect the absorption. This may be the reason why Stephenson & Stickland [1931], who cultured *Cl. sporogenes* on a tryptic digest of caseinogen, failed to observe any $H_2$ absorption.

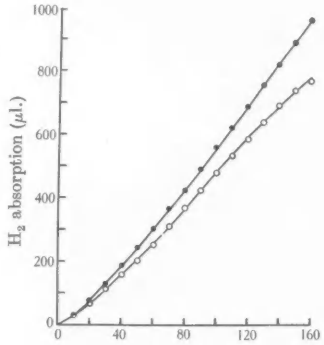



Fig. 1.

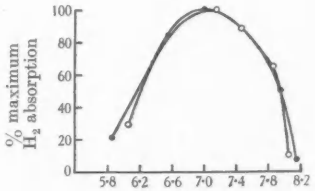



Fig. 2.

Fig. 1. Course of $H_2$ absorption of a washed suspension of *Cl. sporogenes* with glycine (0·2 m.-mol.) and hydroxyproline (0·1 m.-mol.) used as substrates. •—• glycine, o—o hydroxyproline.

Fig. 2. Effect of *p*H on the rate of $H_2$ absorption. o—o hydroxyproline, •—• tryptophan.

The addition of formate to the culture medium also increased the ability of the organisms to activate $H_2$, although much less than did the addition of glucose. When formate was used it was necessary to decrease the peptone concentration in order to induce some formate utilization. The most active bacterial suspensions were obtained from a 16 hr. culture in 3 % neopeptone + 1 % glucose, or from a 36 hr. culture in a 1·5 % neopeptone + 1 % formate, the glucose-containing culture giving by far the best results.[1]

As may be seen from Fig. 2 the optimum *p*H for $H_2$ absorption of a suspension of *Cl. sporogenes* obtained from a 16 hr. culture in 3 % neopeptone + 1 % glucose was found to be about 7·1.

Since the *p*H optima are the same for different substrates, we are probably dealing in each case with the activity of the same enzyme (hydrogenase) which is capable of activating $H_2$. Substrate activation may shift this *p*H optimum slightly, particularly when the substrate activation is low at *p*H 7·1. This *p*H optimum of 7·1 differs considerably from that of the *Bact. coli* hydrogenase or from

[1] It was found necessary to exercise great care in eliminating traces of air from the anaerobic culture jars. Traces of air, which with heavy inocula apparently exert no influence on bacterial growth, very markedly diminished the $H_2$ absorption of bacteria obtained from such a culture. For the same reason it was found necessary to remove $O_2$ traces from buffer and substrate solutions by boiling them just before use.

that of the hydrogenase of sulphate-reducing bacteria with which Stephenson & Stickland [1931] found activity from *p*H 4 to 10 with the optimum at 6·3.

The data in Table I show the results obtained with a large number of biologically important compounds when used as substrates.

Table I. *Relative velocities of hydrogenation of various substrates by gaseous $H_2$ at* p*H 7·1 with a suspension of* Cl. sporogenes, *obtained from a 20 hr. culture in 3 % neopeptone–1 % glucose medium of* p*H 7·4*

The velocity with hydroxyproline is taken as standard (=100).

| 100–50 | | 50–25 | |
|---|---|---|---|
| Hydroxyproline | 100 | Acetylmethylcarbinol | 50 |
| Proline | 100 | Diacetyl | 40 |
| Glycine | 100 | Acrolein | 47 |
| Ornithine | 100 | Acetaldehyde | 33 |
| Arginine | 80 | Glyceric aldehyde | 26 |
| Tryptophan | 67 | | |

| 25–10 | | 10–0 | |
|---|---|---|---|
| Tyrosine | 25 | Acetic acid | 5 |
| Cysteine | 22 | Glutaric acid | 5 |
| Propionic acid | 22 | Glyoxal | 5 |
| Dihydroxyacetone | 22 | Oxalic acid | 4 |
| Glycerol | 19 | Aspartic acid | 2 |
| Glyceric acid | 16 | 2:3-Butylene glycol | 2 |
| Butyric acid | 16 | Malonic acid | 4 |
| Cystine | 16 | | |
| Methionine | 15 | | |

0

| | | |
|---|---|---|
| Leucine | Maleic acid | Acetylurea |
| Isoleucine | Acrylic acid | Oxaluric acid |
| Valine | Fumaric acid | Uric acid |
| Glutamic acid | Glycollic acid | Xanthine |
| Histidine | Lactic acid | Hydantoin |
| Alanine | Hydroxybutyric acid | Formamide |
| Phenylalanine | Tartaric acid | Acetamide |
| Lysine | Tartronic acid | Succinimide |
| Asparagine | Malic acid | Betaine |
| Serine | Glucose | Guanine |
| Formic acid | Formaldehyde | Histamine |
| Succinic acid | Ethanol | Creatine |
| Citric acid | Urea | |

As may be seen in Table I certain amino-acids (hydroxyproline, proline, glycine, ornithine, arginine and tryptophan) are the most active H-acceptors. A similar preference of *Cl. sporogenes* toward amino-acids was found in the detection of H-donators, as reported in a previous publication.

Hydroxyproline, proline and glycine were found by Stickland [1934], ornithine and arginine by Woods [1936] to act as H-acceptors. Their methods and ours give the same results so far as the most powerful H-acceptors are concerned. However, the $H_2$ absorption method used in these experiments adds many less powerful acceptors to the list because of its greater sensitivity.

Many substances other than amino-acids may act as H-acceptors, especially certain aldehydes (acetaldehyde, glyceric aldehyde, acrolein) and ketones (dihydroxyacetone, acetylmethylcarbinol, diacetyl) as well as glycerol, and propionic, butyric and glyceric acids.

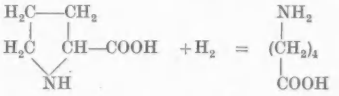
It is worthy of note that many compounds which had been reported [Kocholaty & Hoogerheide, 1938] to act as H-donators, are found to act as H-acceptors too, i.e. ornithine, tryptophan, tyrosine, butyric and glyceric acids, acetaldehyde, glyceric aldehyde, glycerol, and certain others which have a

similar though lesser action. This fact suggests the possibility that an oxidation-reduction between 2 mol. of such a substrate occurs, 1 mol. being oxidized, the other being reduced. Evidence in support of this will be given later.

*Cl. sporogenes* causes fermentation of many substrates. Of the amino-acids tested, serine, arginine and leucine were fermented, as well as the sulphur-containing amino-acids cystine, cysteine and methionine. Of the substances other than amino-acids, glucose and pyruvic acid were strongly fermented. These fermentation reactions take place also in $N_2$, and are independent of the presence of glucose in the culture medium. Some fermentable substrates act also as H-acceptors (arginine, cysteine, cystine, methionine, pyruvic acid) while others do not (leucine, serine, glucose). Although in some cases (cysteine, pyruvic acid) fermentation may be an intermolecular ("Stickland") reaction, in other cases (leucine, serine, glucose) it is highly probable that we are dealing with a true fermentation.

The ultimate fate of substrates acting as H-acceptors is a reduction, but it appears that in most cases the complete reaction is a rather complicated one. As would be expected, the less complicated cases are those in which the substrate acts exclusively as H-acceptor. In cases where the substrate acts as both donator and acceptor, there occur side reactions, reductions not involving the absorption of $H_2$, but the simultaneous dehydrogenation of a second mol. Even in cases where the substrate acts only as H-acceptor, e.g. with proline, hydroxy-proline and glycine, marked mutual differences are observed. Proline and hydroxyproline are reduced through the opening of their ring-bond, without deamination. Glycine, however, is reduced and simultaneously deaminized.

l-*Proline*. The reaction which takes place when *l*-proline acts as H-acceptor with a suspension of *Cl. sporogenes* and $H_2$ as donator is probably:



The $H_2$ absorption was found to be largely independent of the concentration of proline, showing no decrease until the concentration was diminished to 0·006 m.-mol. in 2·5 ml. of suspension. As may be seen from Fig. 3, the absorption becomes less and finally stops completely. As calculated from the amounts of $H_2$ and proline involved the reactants appear to participate in molecular proportions. No $NH_3$ was liberated.

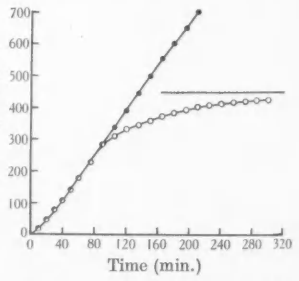



Fig. 3. Course of $H_2$ absorption of a washed suspension of *Cl. sporogenes* with proline (conc. 0·100 m.-mol. (•—•) and 0·020 m.-mol. (o—o) respectively) as substrate.

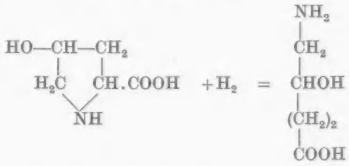
$NH_3$ present at the end of the experiment was estimated by semi-micro steam distillation of the contents of the Warburg vessels after addition of 1 ml. 10 % $Na_2CO_3$. A blank determination was carried out on the same amount of substrate and bacterial suspension. The $NH_3$ was absorbed in 5 ml. $N/70$ $H_2SO_4$ and the excess acid titrated with $N/70$ NaOH.

The product of the reaction (probably δ-amino-*n*-valeric acid) has not been isolated or definitely identified, as there is not a satisfactory method for the identification of this substance in such small amounts. However, since Stickland [1935] showed that when proline acts as H-acceptor

with alanine as a donator deamination does not occur, the ring being opened and the proline reduced to δ-amino-*n*-valeric acid, it is probable that in this case a similar reaction occurs.

l-*Hyroxyproline*. The reaction which takes place when hydroxyproline acts as H-acceptor is similar to that occurring with proline. It was shown as before that 1 mol. hydroxyproline absorbs 1 mol. $H_2$, and that no $NH_3$ is liberated.

Therefore the reaction taking place is probably:



Here again no suitable method was found for the isolation and identification of such small amounts of γ-hydroxy-δ-aminovaleric acid.

*Glycine*. As has already been mentioned glycine, unlike proline and hydroxyproline, is deaminated during reduction. One mol. glycine absorbs 1 mol. $H_2$, liberating 1 mol. $NH_3$.

The reaction which takes place is therefore:

$$H_2N.CH_2.COOH + H_2 = CH_3.COOH + NH_3.$$

A separate set of experiments showed that 1 mol. glycine yielded 1 mol. of a volatile acid, which was definitely identified as acetic acid.

The contents of the Warburg vessels, after the completion of the experiment, were transferred into a micro steam distillation apparatus and 2 ml. 4*N* $H_2SO_4$ were added. After distillation (*c.* 50 ml.) the amount of volatile acid was estimated by titration with *N*/70 NaOH, after removal of $CO_2$ by heating to 100°. Blank determinations with a known amount of acetate showed that the whole amount of acetic acid had thus been accounted for. For the identification of acetic acid, the Feigl test [1937] was used. We wish to express our thanks to the Microchemical Department Institute for the identification of this compound.

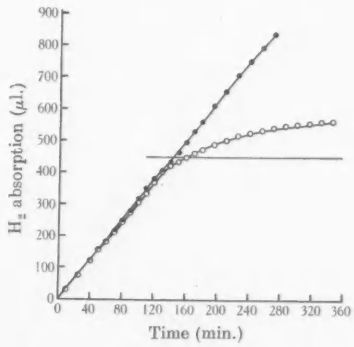



Fig. 4. Course of $H_2$ absorption of a washed suspension of *Cl. sporogenes* with glycine (conc. 0·100 (•—•) and 0·020 (o—o) m.-mol. respectively) as substrate.

As may be seen from Fig. 4, the $H_2$ absorption does not cease when all the glycine has disappeared but continues slowly, finally exceeding the theoretical

limit. This is due in some degree to the action of acetic acid as H-acceptor. The slope of the line of $H_2$ absorption for acetic acid is quite different from that for glycine, no difficulty being encountered in determining the end-point of the reaction.

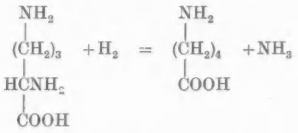
There was further evidence that another side reaction occurred, but only to a very small extent. It was probably as indicated by the equation:

$$4NH_2.CH_2.COOH + 2H_2O = 4NH_3 + 3CH_3.COOH + 2CO_2.$$

There was a slight development of $CO_2$, and of $NH_3$ and acetic acid in the ratio of 4:3. It is doubtful if this reaction plays a very important role.

d-*Ornithine*. As was reported in our previous publication [Kocholaty & Hoogerheide, 1938], ornithine acts as a feeble H-donator. On the other hand it may be activated as a powerful H-acceptor (Table I). This is probably why the reaction with ornithine as the only substrate in $H_2$ is more complicated than that obtained by using the previously mentioned substrates. Of the two amino groups present in the ornithine molecule, only one is split off. This is in agreement with the results of Woods [1936], who found that when ornithine reacts with the donator alanine it accepts two H atoms and undergoes reductive deamination to δ-amino-*n*-valeric acid.

The evidence suggests that a similar reaction occurs when gaseous $H_2$ acts as H-donator, the main reaction probably being as indicated by the equation:



the first reaction product being the same as is formed by the reduction of proline.

When a surplus of ornithine was present and the reaction was stopped at a point where there was still an active $H_2$ absorption, it was found that considerably more $NH_3$ had been formed than is indicated by the above equation. This was due to secondary reactions also observed by Woods [1936]. The nature of these secondary reactions is not known, but they are probably oxidation-reduction reactions in which one mol. ornithine is oxidized while another mol. is being reduced. This explanation agrees with the fact that ornithine may act either as H-acceptor or donator.

When ornithine was incubated in $N_2$ with a suspension of *Cl. sporogenes* there occurred a slight but uniform production of $CO_2$ with considerable amounts of $NH_3$.

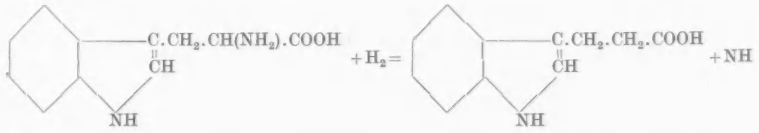
When a surplus of ornithine is present, the quantity of $NH_3$ formed from ornithine in $H_2$ equals that calculated from the $H_2$ absorption according to the above equation, plus the quantity split off in the same time in $N_2$.

The reaction occurring in $N_2$ is not clearly understood. The ratio of $CO_2$ to $NH_3$ is not constant, but increases with increasing concentration of ornithine. With 0·015 m.-mol. ornithine the ratio was very nearly 1:5, but when 0·05 m.-mol. was used the ratio was almost 1:1. This indicates that we are not dealing with a single reaction.

In the later stages of the reaction when the ornithine has been completely deaminized, $H_2$ absorption continues, but at a much lower rate (Fig. 5). It is probable that one of the products of the oxidation-reduction reaction then acts as a substrate, being reduced without deamination.

l-*Tryptophan.* Tryptophan, found to be an active H-acceptor, is also an active H-donator. Since it may act either as H-acceptor or donator secondary reactions would be expected to occur even more than with ornithine.

The principal reaction which occurs with this compound as H-acceptor and gaseous $H_2$ as the donator is probably:




The presence of indolepropionic acid was demonstrated by extraction with ether and precipitation as a nitroso-compound.

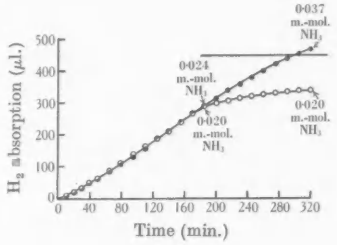



Fig. 5.

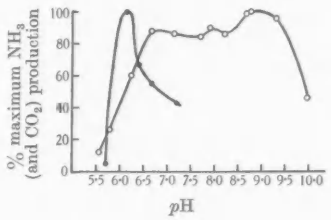



Fig. 6.

Fig. 5. Course of $H_2$ absorption of a washed suspension of *Cl. sporogenes* with ornithine as substrate (conc. 0·040 (•—•) and 0·020 (o—o) m.-mol.).

Fig. 6. Effect of *p*H on anaerobic decomposition of pyruvic acid and tryptophan by washed suspensions of *Cl. sporogenes*, measured as $CO_2$ and $NH_3$ respectively. •—• pyruvic acid, o—o tryptophan.

The absorption of $H_2$ by tryptophan usually ceases when about $\frac{1}{3}$–$\frac{1}{2}$ of that calculated for the above equation has been absorbed. $NH_3$ estimation shows, however, that all of the tryptophan has been deaminized.

In $N_2$ tryptophan is readily decomposed, 1 mol. tryptophan yielding approximately 1 mol. $NH_3$ in addition to small amounts of $CO_2$ and considerable amounts of indolepropionic acid. The reaction is in general agreement with the equation:

$$2 \text{ tryptophan} = 2NH_3 + 1 \text{ indolepropionic acid} + 1 \text{ indolepyruvic acid.}$$

Approximately 1 mol. indolepropionic acid was formed to 2 mol. $NH_3$ during anaerobic decomposition of tryptophan *in vacuo* or in $N_2$. Indolepyruvic acid, although not definitely identified, is probably the source of the small amount of $CO_2$ formed during anaerobic decomposition.

Very little influence of the *p*H on the rate of anaerobic decomposition of tryptophan was found inasmuch as there exists a broad optimum from *p*H 6·7 to 9·0 (Fig. 6). The optimum for $H_2$ absorption, however, was *p*H 7·0, the activity decreasing considerably on each side of this value.

*Fermentable substrates*

No $H_2$ uptake occurred when serine was mixed with a suspension of *Cl. sporogenes* in an atmosphere of $H_2$; instead carbon dioxide and considerable amounts of ammonia were produced as a result of fermentation. This fermentation proceeds equally well in $N_2$.

When the sulphur-containing amino-acids, cysteine, cystine or methionine were used, active fermentation occurred with $H_2S$ as the chief gaseous product. While with cysteine and methionine fermentation begins immediately and continues at a uniform speed, gas development with cystine shows a considerable lag period. Contrary to the results with serine much $H_2$ is absorbed in an atmosphere of $H_2$ by these sulphur-containing amino-acids. This was true also for arginine. Although a good H-acceptor, fermentation occurs simultaneously with the production of $CO_2$ and $NH_3$. The fermentation, however, decreases rapidly in a short time with little utilization of the substrate.

Of compounds other than amino-acids, pyruvic acid and glucose are actively fermented under the conditions of these experiments. The optimum *p*H for the fermentation of pyruvic acid was found to be 6·2 (Fig. 6). This acid yields 1 mol. $CO_2$ per mol. of the acid, while among the degradation products approximately 0·5 mol. of a volatile acid was detected which was very probably acetic acid, since it gave a positive iodo-lanthanum reaction.

Glucose was the only substrate used with which much $H_2$ was produced.

Recently Woods & Clifton [1937] have shown that *Cl. tetanomorphum* decomposes many amino-acids with the liberation of $H_2$. *Cl. sporogenes* behaves very differently in this respect inasmuch as none of the amino-acids tested but only glucose yielded $H_2$.

## Discussion

Stephenson & Stickland [1931] suggest a correlation between the ability of certain species of micro-organisms to activate gaseous $H_2$ and their ability to ferment formic acid, only those species which ferment formic acid being able to activate $H_2$.

Since *Cl. sporogenes* in general does not ferment formic acid it was considered to be unable to activate $H_2$. This theory does not appear to be valid under all conditions in the case of *Cl. sporogenes* since formic acid is not fermented even by suspensions of the organisms which are highly active so far as H-activation is concerned. Although formate has usually been considered to be the precursor of $H_2$ produced by bacteria, evidence has been obtained recently which suggests that $H_2$ may arise by some mechanism other than by way of formate [Stephenson & Stickland, 1932; Stephenson, 1937; Woods & Clifton, 1937]. It seems to us, therefore, that there is more evidence of a correlation between the ability of certain bacterial species to produce $H_2$ gas, and the ability to activate $H_2$ gas, independently of its formation from formate or by some other mechanism. This appears more probable since the reversibility of the reaction $2H = H_2$ was clearly demonstrated with a suspension of *B. coli* by Green & Stickland [1934] and Woods [1936].

In many cases, certain specific bacterial enzymes are produced only when the organisms require them for the utilization of a particular substrate (adaptive enzymes). It is possible that hydrogenase is present in the resting *Cl. sporogenes* only when the organisms are cultured in a medium in which $H_2$ development occurs.

The production of $H_2$ from glucose and the utilization of $H_2$ with certain amino-acids by the same suspension is an example of a possible linking of carbohydrate and protein metabolism by this particular species.

The results obtained with the redox indicator method (used by Stickland for the detection of H-acceptors) and those obtained by using gaseous $H_2$ are comparable, since the latter method detects all the H-acceptors found by the indicator method but, in addition, it reveals many less potent H-acceptors, and thus is superior to the redox indicator method. It is applicable however only in a *p*H range in which $H_2$ is activated (*p*H 6–8).

A sharp distinction between H-donators and H-acceptors can no longer be maintained. Many substrates act as donators as well as acceptors, giving an intermolecular oxido-reduction, one molecule being oxidized, another reduced.

Fermentation reactions, Stickland reactions and these intermolecular oxido-reductions, appear to be the main sources of energy of *Cl. sporogenes*. The very decided preference of this organism for amino-acids is in accordance with its putrefying action on proteins.

This work indicates that certain amino-acids, such as *d*-lysine, glutamic acid and aspartic acid, are not utilized by *Cl. sporogenes* to any extent, since they are not fermented, and play no important role as H-acceptors or H-donators.

## Summary

1. Washed suspensions of *Cl. sporogenes* which had been grown on culture media containing glucose were found to be able to utilize gaseous $H_2$.

2. This ability to utilize $H_2$ was used as the basis of a method for the detection of substrates able to act as H-acceptors for this organism.

3. Proline, hydroxyproline, glycine, ornithine, arginine, tryptophan, certain aldehydes and ketones and to a lesser extent many other substrates may act as H-acceptors with $H_2$ as donator.

4. The chemical reactions concerned in the reduction of certain of these acceptors have been studied. Evidence is given that each of the 6 amino-acids mentioned absorbs 1 mol. $H_2$ for each mol. amino-acid. Except for proline and hydroxyproline, which are reduced by the opening of the ring bonds, they all undergo reductive deamination.

5. Several substrates act as donators as well as acceptors, and hence give rise to oxido-reduction reactions in which one mol. is oxidized and another is reduced. These reactions are independent of the presence of $H_2$, and occur also as side-reactions accompanying $H_2$ uptake.

6. Serine, cysteine, cystine, methionine, pyruvic acid, glucose and to a lesser extent arginine, are actively fermented by washed suspensions of *Cl. sporogenes*. Glucose was the only substrate from which $H_2$ was evolved.

The authors wish to express their thanks to Dr Ellice McDonald, Director, for his interest and support throughout this work.

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# CXXIX. SYNTHESIS OF VITAMIN $B_1$ BY INTESTINAL BACTERIA OF THE RAT

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(*Received 6 April 1938*)

Many types of micro-organisms have been tested by various workers for their ability to synthesize "vitamin B" (=complex). In such studies the organisms were grown in various nutrient media and the "vitamin B" was detected by its growth-promoting action or by curative tests on experimental animals such as the rat, the mouse or the pigeon. Thus "vitamin B" has been reported to have been produced by Pfeiffer's bacillus [Damon, 1923], *Mycobacterium smegmatis*, *Mycobact. phlei* and *Mycobact. moelleri* [Damon, 1924], *Monila candida* [Hoet *et al.* 1924], *B. vulgatus* [Scheunert & Schieblich, 1923], various types of *Torula*, *Oospora*, *Actinomyces* and *Eubacteriales* [Sunderlin & Werkman, 1928], *B. ellenbachiensis* and *B. prodigiosum* [Schieblich, 1931], *B. lactis aerogenes* and *Vibrio alcaligenes* [Schieblich, 1933]. On the other hand, the following micro-organisms (among others) have been found to produce no vitamin: *Lactobacillus bulgaricus* and *Amylomucor* B [Wollman, 1921; Wollman & Vagliano, 1922], *Bact. lactisacidi*, *B. mycoides*, *B. subtilis* and *Micrococcus agilis* [Slanetz, 1923], *Bact. coli* [Cooper, 1914], the tubercle bacillus [Cunningham, 1924].

Considering the above results together, it seems reasonable to believe that different types of bacteria may vary in their ability to synthesize "vitamin B". This view receives direct support from the experiments of Schieblich [1931], who showed that there was a well marked difference in the extent of the synthesis of "vitamin B" by various bacteria. Thus, when grown in a suitable culture medium, *B. vulgatus* was found to produce large amounts of the vitamin B complex, *B. mycoides* smaller amounts and *B. mesentericus* very small quantities only. These conclusions were based on growth tests on rats.

An examination of the literature also shows that studies of the synthesis of "vitamin B" by a specific organism have produced somewhat contradictory results. To take one example, *Bact. coli* was reported to be unable to synthesize "vitamin B", by Cooper [1914], Damon [1921], Eijkman *et al.* [1922], and Weill *et al.* [1922], while positive results were claimed by Kuroya & Hosoya [1923] and by Sunderlin & Werkman [1928]. Of the various causes which may account for this lack of agreement the following are perhaps the most important: (1) the use of different methods for detecting the presence of the vitamin, (2) the administration of insufficient amounts of micro-organisms for the feeding test and (3) the use of different culture media by the various workers.

Most of the work mentioned above dealt probably with the bacterial synthesis of the "vitamin B" complex as a whole rather than with its separate components. More recently, however, the synthesis of vitamin $B_1$ by *B. vulgatus* has been reported by Guha [1932]. Various other investigators have presented evidence which suggested that vitamin $B_1$ is synthesized in the intestinal tract of the rat [Roscoe, 1931; Guerrant *et al.* 1935; Whipple & Church, 1935; Leong, 1937].

The work described in this paper was designed: (1) to study the extent of the synthesis of vitamin $B_1$ by the intestinal bacteria of the rat when grown in broth medium and (2) to determine whether the synthesized vitamin is secreted by the organisms into the surrounding culture medium or is retained within the bacteria. Employing the bradycardia method for the assay of vitamin $B_1$ [Drury & Harris, 1930; Birch & Harris, 1934; Leong & Harris, 1937], data concerning the rate and the extent of this bacterial synthesis in terms of the International Unit have been obtained.

## 1. Rate and extent of synthesis of vitamin $B_1$ by intestinal bacteria

*Material and method.* As culture medium ordinary bullock's heart extract broth (*p*H 7·2) was used. We found that this broth medium contained traces of vitamin $B_1$, the exact amount of which was determined after adsorption on acid clay in accordance with the technique previously described by Harris & Leong [1936]. This factor did not vitiate our results, as we carried out quantitative estimations of vitamin $B_1$ in the medium both before and after inoculation. However, a separate experiment was undertaken in which a specially prepared vitamin-$B_1$-free broth was used for growing the bacteria, with the object of confirming the results of our main experiment. It was thought advisable to employ ordinary broth in the main experiment in preference to a specially devitaminized broth, as the former would perhaps procure a more natural growth of the bacteria.

The organisms were obtained from the pooled caecal contents of three adult piebald rats. The animals weighed about 200 g. (about 6 months old) and were reared on the laboratory stock diet, consisting mainly of brown bread, meat, cabbage and milk.

*Bacteriological examination of caecal contents.* Microscopic and cultural examination of the caecal contents used for inoculation showed the presence of the following organisms: (1) enterococci, about 5%; (2) lactobacilli, about 1%; (3) various clostridia which presumably did not grow to any great extent in our aerobic mixed cultures; (4) organisms of the *Bact. coli* group, which constituted the rest.

*Preliminary experiments.* These need not be detailed here and were undertaken to find the most convenient quantities of culture medium to provide an amount of vitamin $B_1$ suitable for assay by the bradycardia method. It was found that 150 ml. of broth constituted a suitable amount. Our specimens were prepared and analysed in triplicate in order to obtain more reliable results.

### *Main experiment*

Three rats were killed by coal gas, their caecal contents were mixed together and made into a thin suspension in sterile saline. After this suspension had been left to stand for $\frac{1}{2}$ hr. to allow coarse particles to settle, 21 sterilized flasks of 250 ml. capacity (each containing 150 ml. broth) were each inoculated with 1 ml. of the supernatant fluid and incubated at 37°. The cultures were allowed to grow for $\frac{1}{2}$, 1, 2, 3, 4, 6 or 9 days, and after each period 3 flasks were removed and the bacterial mass and supernatant liquid in each specimen were assayed separately for vitamin $B_1$. The cultures were centrifuged at 4000 r.p.m. for 1 hr. and the deposited bacteria were dried *in vacuo* at room temperature over $P_2O_5$. The dried deposits were then weighed and assayed. The supernatant liquids were passed through a Seitz filter and the *p*H was determined colorimetrically.

The vitamin $B_1$ content of each specimen of supernatant liquid was determined by the bradycardia method after adsorption of the vitamin on acid clay. The results of this experiment are given in Table I and depicted in Fig. 1.

Table I. *Synthesis of vitamin $B_1$ by intestinal bacteria grown in 150 ml. broth medium*

| Days of incubation | | Cure of bradycardia, days (individual tests) | Vitamin $B_1$ content, I.U. (average of tests) | Total vitamin $B_1$ content in culture and supernatant liquid, I.U. |
|---|---|---|---|---|
| | Sterile broth (*p*H 7·2) | 3, 3, 3, 5 | 1·6 | — |
| ½ | Culture | 2, 3, 4 | 1·3 | 2·6 |
| | Supernatant liquid (*p*H 7·3) | 1, 3, 4·5 | 1·3 | |
| 1 | Culture | 3, 4, 5·5 | 1·9 | 3·7 |
| | Supernatant liquid (*p*H 7·5) | 3, 4, 4·5 | 1·8 | |
| 2 | Culture | 3, 4, 4·5 | 1·7 | 2·5 |
| | Supernatant liquid (*p*H 8·1) | 1, 2, 2·5 | 0·8 | |
| 3 | Culture | 2, 3, 3 | 1·1 | 2·4 |
| | Supernatant liquid (*p*H 8·4) | 3, 3, * | 1·3 | |
| 4 | Culture | 1, 1, 2 | 0·5 | 1·8 |
| | Supernatant liquid (*p*H 8·4) | 2, 2, 5 | 1·3 | |
| 6 | Culture | 0·5, 1, 1·5 | 0·4 | 1·2 |
| | Supernatant liquid (*p*H 8·6) | 1, 3, * | 0·8 | |
| 9 | Culture | 0·5, 0·5 | 0·2 | 0·6 |
| | Supernatant liquid (*p*H 8·6) | 1, 1 | 0·4 | |

* Test rat died.

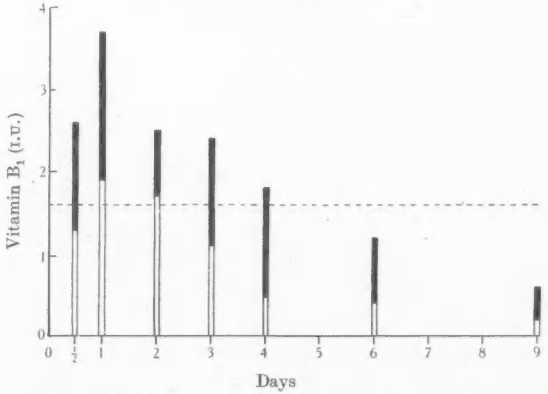


Fig. 1. Synthesis of vitamin $B_1$ in broth medium. ■ Amount of vitamin $B_1$ in supernatant liquid. □ Amount of vitamin $B_1$ in dried bacterial growth. ---- Amount of vitamin $B_1$ in original sterile medium.

*Rate of synthesis of vitamin $B_1$.* It will be seen from Fig. 1 that there has been an appreciable synthesis of vitamin $B_1$ in the ½, 1, 2 and 3-day cultures, as the combined vitamin $B_1$ content in each bacterial growth and its supernatant fluid exceeded that originally present in the sterile medium. The combined vitamin content of the 4-day culture and its supernatant liquid was not appreciably higher than that present in an equivalent amount of the sterile medium.

These results demonstrate that the quantity of the vitamin found in the different cultures varied with the age of the culture. Under the conditions of this experiment, the greatest synthesis of the vitamin occurred during the first 3 days of incubation. When this period was exceeded the amount of vitamin $B_1$ in the bacterial growth was found to be much smaller. Inspection of Fig. 1 will show that there had been a distinct diminution in vitamin $B_1$ content after the 4th day of incubation.

*Amount of vitamin $B_1$ in bacteria.* The dry weight and the vitamin $B_1$ content of each bacterial mass are shown in Table II. The weights of the bacterial growths after varying periods of incubation were found to be fairly constant,

Table II. *Vitamin $B_1$ content of cultures of intestinal bacteria*

| Age of culture days | Dry wt. of culture g. | Cure of bradycardia, days (av. of 3 tests) | Vitamin $B_1$ in dried bacteria I.U./g. |
|---|---|---|---|
| ½ | 0·12 | 3·0 | 11 |
| 1 | 0·12 | 4·2 | 16 |
| 2 | 0·13 | 3·8 | 13 |
| 3 | 0·13 | 2·7* | 9 |
| 4 | 0·13 | 1·3 | 4 |
| 6 | 0·12 | 1·0* | 3 |
| 9 | 0·11 | <0·5* | <2 |

* Average of 2 tests.

i.e. about 0·13 g. It will be noted that in the 1-day culture, the dried bacteria contained about 16 I.U. of vitamin $B_1$ per g. while the vitamin content of the cultures was found to decrease when grown for longer periods. Thus the 9-day culture contained less than 2 I.U. per g.

It is realized that the cultures examined in these studies consisted of a mixture of many types of micro-organisms. It is of interest to compare the vitamin $B_1$ potency of such intestinal bacteria (the richest sample containing about 16 I.U. per g.) with that of yeast, which is perhaps the richest known natural source of this vitamin. The highest values to be found in the literature for yeast are 15 I.U. per g. (quoted from Cowgill, 1934, and based on the relationship that 20 "milligram-equivalents" equal 1 I.U.) and 23 I.U. per g. [Baker & Wright, 1935].

*Destruction of vitamin $B_1$ during incubation.* It will be seen from Fig. 1 that the vitamin $B_1$ content of the bacterial growth remained at a high level for the first 3 days of incubation, and then decreased rapidly until less than 0·2 I.U. was found in the 9-day growth. There was also a distinct decrease in the amount of vitamin $B_1$ in the supernatant liquids when the time of incubation was longer than 6 days. These observations suggest that a destruction of the vitamin had occurred when the micro-organisms were grown for periods longer than 3 days. A destruction of the vitamin during the first 3 days might also have occurred, although it was not possible to demonstrate this point. The following factors may have accelerated the destruction of the vitamin: (1) the high temperature of incubation and (2) the increasing alkalinity of the culture media as a result of bacterial metabolism. In Table I are given the figures showing the gradual rise in *p*H values of the supernatant liquids as the period of incubation was prolonged.

2. Retention of the synthesized vitamin $B_1$ in the bacterial cells

An interesting question is whether the synthesized vitamin can diffuse out of the bacterial cells into the culture medium. In a study of the formation of vitamin $B_1$ by *B. vulgatus*, Guha [1932] showed that the synthesized vitamin was not secreted into the surrounding medium (as judged by curative tests on pigeons). The following experiment was carried out in an attempt to obtain information on this point with regard to intestinal bacteria.

*Medium.* Ordinary broth was brought to *p*H 10 by the addition of NaOH and autoclaved for 4 hr. under a pressure of $1\frac{1}{2}$ atmospheres. The broth was then readjusted to *p*H 7·2 by the addition of conc. HCl. It was hoped to destroy all the vitamin $B_1$ in the broth by this treatment. The results of several separate determinations showed that this autoclaved broth contained no appreciable amount of vitamin $B_1$ (less than 0·4 I.U. was found in 500 ml.).

*Method.* Six 150 ml. samples of this medium were then inoculated with a suspension (in normal saline) of the caecal contents from a normal piebald rat. These were incubated aerobically at 37° and examined for vitamin $B_1$ after intervals of 1 and 3 days. Triplicate samples of the 1-day culture were taken and the bacterial growth was separated from the supernatant liquid by centrifuging followed by filtration through a Seitz filter as before. Each culture was assayed separately. In testing the supernatant liquids the filtrates from the 3 specimens were combined, made up to the original volume (i.e. 450 ml.) with water, and adsorbed on acid clay. A similar procedure was adopted for the 3-day cultures.

*Results.* The results are shown in Table III. It was found that during the period of incubation (i.e. 1–3 days) no detectable amounts of vitamin $B_1$ had appeared in the supernatant liquid. At the same time, however, a synthesis of

Table III. *Synthesis of vitamin $B_1$ by intestinal bacteria grown in vitamin-$B_1$-free broth*

| Period of incubation days | | Cure of bradycardia, days (individual tests) | Vitamin $B_1$ content I.U. |
|---|---|---|---|
| | Sterile broth 500 ml. (vitamin $B_1$-free) | <1, <1, <1 | <0·4 |
| 1 | Culture* | 1, 1·8, 3·5 | 0·8 |
| | Supernatant liquid, 450 ml. | <1 | <0·4 |
| 3 | Culture* | 4, 4, 6 | 2·2 |
| | Supernatant liquid, 450 ml. | <1 | <0·4 |

* Culture from 150 ml. medium.

the vitamin had occurred, for the separated bacterial growths were found to contain demonstrable amounts (0·8 I.U. in the 1-day culture and 2·2 I.U. in the 3-day culture). These observations suggest that there was no diffusion of vitamin $B_1$ from the bacterial cells into the surrounding medium although the intestinal bacteria of the rat could synthesize vitamin $B_1$ in broth which was practically free from it.

The question may be raised as to why the rat develops $B_1$ avitaminosis when the vitamin can be synthesized by its intestinal flora. Our experiments indicate that no diffusion of the vitamin out of the bacterial cells takes place, so that no absorption would be expected from the caecum of the rat. When the bacterial growth was fed to the animal it was then able to digest and absorb the vitamin.

SUMMARY

Quantitative studies of the synthesis of vitamin $B_1$ (in terms of the International Standard) by intestinal bacteria grown in ordinary broth medium have been made.

Under the conditions described, the greatest amount of vitamin $B_1$ was formed in the 1-day culture. The richest culture was found to contain about 16 I.U. per g. A destruction of the vitamin was observed when the micro-organisms were grown for periods longer than 3 days.

No diffusion of synthesized vitamin $B_1$ from the bacterial cells into the broth medium could be detected.

We wish to thank Prof. H. R. Dean and Dr L. J. Harris for their kind interest and helpful criticism.

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# CXXX. VITAMIN A AND THE THYROID

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(*Received 21 April 1938*)

There is a considerable amount of evidence indicating that the action of thyroxine on metabolism is antagonized by vitamin A or by carotene. The effect was first observed by Euler & Klussmann [1932], who found that carotene partly protected rats from losing weight on the administration of thyroxine. They also obtained indications that thyroxine reduced the storage of vitamin A and carotene in the liver. Small numbers of animals were used in these experiments and it is unfortunately true of many other publications on this subject that the experimental procedure can be adversely criticized on similar grounds; Abelin [1933], for example, used only two guinea-pigs in each group when he studied the effect of thyroxine on the fate of carotene injected intraperitoneally. Nevertheless, a survey of the literature as a whole leaves the impression that a case has been made out for the view that there is antagonism between the actions of vitamin A and thyroxine.

Some of the more significant observations may be noted. Chevalier & Baert [1934] found that the basal metabolic rate of rats tended to rise on vitamin A depletion and to fall below normal when large doses were given. Abelin [1935, 1] showed that appropriate dosage with the vitamin will counteract to a marked extent the influence of thyroxine on the B.M.R., and his observations were confirmed by Rappai & Rosenfeld [1935]. Fasold & Peters [1933] found that they could relieve symptoms of thyroid overdosage by administering vitamin A and *vice versa*. Fleischmann & Kann [1936] found that vitamin A antagonized the effect of thyroxine on acetonitrile poisoning. Perhaps the most striking claims are those which record improvement in patients suffering from thyrotoxicosis after treating with large doses of vitamin A [Wendt, 1935; Dietrich, 1936].

In the course of a study of the factors influencing storage of vitamin A in the liver and its mobilization it was necessary to investigate the influence of the thyroid, particularly because there is a certain amount of evidence, not entirely convincing, it must be admitted, in the literature that thyroxine administration causes a depletion of the vitamin A reserves [Abelin, 1933; Fasold & Peters, 1933; Wendt, 1935 etc.]. The opportunity was taken to make some studies of the influence of thyroxine and vitamin A on the metabolic rate, using sufficient animals to yield significant results.

## Experimental

Young rats of 68–80 g. weight from a homozygous strain (Glaxo-Wistar) were divided into three groups. All were fed on a basal "A-free" diet consisting of casein, 10; meat-meal, 10; wheat-flour, 59·5; yeast extract (yeastrel), 8; Hubbell *et al.* [1937] salt mixture No. 351, 2·5; and olive oil 10 parts. One group, 32 in number, served as a control. The 35 animals of another received twice weekly a dose of 35 I.U. vitamin A, which was estimated to be a little more than their requirements for growth, whilst the third group, consisting of 37 rats, received 3000 I.U. daily.

[1] Holder of a Rockefeller Foundation Fellowship.

Table I. *Basal metabolism ml. $O_2$/kg./min.*

All figures represent the mean of triplicate estimations. The figures in brackets are the reference numbers of individual animals

| Group | Vitamin A-free diet | | | | 10 I.U. vitamin A daily | | | | 3000 I.U. vitamin A daily | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Control | (76) 22·84<br>(94) 21·78<br>(102) 21·54 | | | | (54) 21·09<br>(61) 21·83<br>(49) 21·99) | | | | (1) 22·48<br>(27) 22·82<br>(29) 21·64 | | | |
| | (*a*) | Injections* | (*b*) | % rise | (*a*) | Injections* | (*b*) | % rise | (*a*) | Injections* | (*b*) | % rise |
| Thyroxine: | (73) 21·3 | 7 | 26·23 | 23·1 | (52) 20·6 | 7 | 28·42 | 37·9 | (2) 23·33 | 7 | 29·11 | 24·8 |
| (*a*) before, | | 9 | 31·16 | 46·2 | | 8 | 28·94 | 40·4 | | 7 | 28·16 | 20·7 |
| (*b*) after | | 11 | 33·05 | 55·1 | | | | | | 8 | 26·82 | 15·8 |
| | (74) 22·6 | 7 | 25·93 | 14·7 | (68) 23·3 | 7 | 33·58 | 44·1 | | 9 | 26·64 | 14·1 |
| | | 11 | 37·62 | 66·4 | | 11 | 38·0 | 63·1 | (3) 22·64 | 7 | 24·41 | 7·6 |
| | (100) 21·44 | 7 | 27·15 | 26·1 | (69) 20·66 | 7 | 35·08 | 69·8 | | 9 | 28·29 | 24·9 |
| | | 9 | 34·10 | 58·6 | | 9 | 31·24 | 51·2 | | 11 | 31·72 | 39·9 |
| | | 11 | 35·90 | 67·4 | | | | | (15) 23·24 | 7 | 27·25 | 17·2 |
| | | | | | | | | | | 9 | 29·09 | 25·2 |
| | (*a*) | | (*b*) | % rise | (*a*) | | (*b*) | % rise | (*a*) | | (*b*) | % rise |
| Dinitrophenol: | (93) 21·95 | | 32·38 | 47·5 | (80) 20·48 | | 28·12 | 37·3 | (28) 22·88 | | 35·14 | 53·5 |
| (*a*) before, (*b*) after, | (95) 22·87 | | 31·64 | 38·1 | (67) 21·82 | | 32·56 | 49·2 | (16) 22·48 | | 30·87 | 37·3 |
| 5 mg. per kg. | (101) 22·78 | | 34·32 | 50·6 | (62) 23·38 | | 30·08 | 28·6 | | | | |
| Dinitrophenol: | (93) 21·95 | | 41·82 | 90·4 | (67) 21·82 | | 46·87 | 114·8 | (17) 23·01 | | 40·00 | 73·8 |
| (*a*) before, (*b*) after, | (101) 22·78 | | 41·15 | 80·7 | | | | | (28) 22·88 | | 44·97 | 96·5 |
| 10 mg. per kg. | | | | | | | | | (16) 22·48 | | 49·32 | 119·3 |

* Injections of 100 $\gamma$.

The vitamin was supplied in the form of a concentrate containing approximately 1,700,000 I.U. per g. A measured volume of a solution of this in olive oil was administered to the animals daily from an "Agla" micro-syringe.

*Basal metabolic rate*

Basal metabolism determinations were made after the animals had reached a weight of about 150 g. Most of them attained this weight in 30 days. The apparatus and method described by Richards & Collison [1928] were found very suitable for the purpose. After 80 days on the diets the rats in each group were divided into three subgroups. One lot were left as they had been to serve as controls. Another group was given 19 doses of 100$\gamma$ of thyroxine spread over 28 days, administration being by subcutaneous injection. The third group received subcutaneous injections of 2:4-dinitrophenol, 11 injections at the level of 5 mg. per kg. and subsequently 4 injections at 10 mg. per kg. Basal metabolism studies were made on these animals, care being taken in the case of those dosed with dinitrophenol to make the determinations about half an hour after an injection. Food was withheld for 18 hr. before a B.M.R. determination was made. Representative results are shown in Table I.

The figures show a clear effect of heavy dosage with vitamin A in counteracting the effect of thyroxine treatment. No such action was detectable when the intake of vitamin was just sufficient to supply the requirements of the animal. There is no evidence of such an antagonistic action between the vitamin and 2:4-dinitrophenol. This is an additional piece of evidence differentiating the action of thyroxine and that of the thermogenic drugs of the dinitrophenol class. The latter are known to act by influencing directly the oxidative mechanisms of the cells, whereas the precise mode of action of the thyroid hormone still remains obscure.

The results of the B.M.R. estimations are supported by records of the effects of the various treatments on the weight of the animals. The figures in Table II confirm the observations of other workers who have recorded that vitamin A tends to protect against the loss of weight which thyroxine treatment causes.

Table II. *Change of weight in g. in 28 days*

| Vitamin A I.U. per day | | Control | Thyroxine | Dinitrophenol |
|---|---|---|---|---|
| Nil | ♂ | + 7·7 | −11 | − 3·3 |
| | ♀ | + 7·1 | − 1·6 | + 3·0 |
| 10 | ♂ | +15·7 | −17·5 | +13·0 |
| | ♀ | +11·6 | + 0·4 | + 8·2 |
| 3000 | ♂ | +21·0 | − 8·6 | +13·4 |
| | ♀ | + 7·0 | + 2·6 | + 9·6 |

The possibility that a response by the adrenal cortex is evoked by prolonged administration of large doses of vitamin A is now being investigated.

*Storage of vitamin A in the liver*

All the animals employed in this experiment were killed and the reserves of vitamin A in the liver estimated by measurement of the intensity of selective absorption in the ultra-violet at 328m$\mu$. The livers of the animals which had been fed on the "A-free" diet for 110 days were found, as was expected, to contain no measurable amount of the vitamin. The second group of animals, each of which received 10 I.U. vitamin A daily, also showed no detectable

storage, presumably because that amount was just about the minimum required for satisfactory growth.

The third group, dosed with the larger amount of 3000 I.U. per day, showed storage. The estimations are set out in Table III.

Table III. *Rats reared on diet supplying 3000 I.U. vitamin A daily*

I.U. × 1000 present in whole liver

| | | Controls | Thyroxine | Dinitrophenol |
|---|---|---|---|---|
| | ♂ | 75·7 | 79·2 | 102·0 |
| | | 65·5 | 134·4 | 105·6 |
| | | 106·8 | 103·3 | 103·3 |
| | | 64·8 | 111·0 | 91·8 |
| | | 57·0 | 76·0 | 88·4 |
| | | 55·2 | 72·7 | 93·8 |
| | | — | 79·2 | 78·6 |
| | Mean | 70·8 | 93·7 | 94·8 |
| | ♀ | 52·8 | 80·3 | 100·8 |
| | | 64·8 | 61·5 | 81·6 |
| | | 66·6 | 85·2 | 66·6 |
| | | 68·0 | 77·5 | 84·0 |
| | | 33·6 | 81·3 | 84·0 |
| | | 67·2 | — | — |
| | Mean | 58·8 | 77·2 | 83·4 |

Calculated on the basis of the amount of vitamin A in 100 g. of liver the differences are still apparent.

I.U. × 1000 vitamin A per 100 g. liver

| Control | | Thyroxine | | Dinitrophenol | |
|---|---|---|---|---|---|
| ♂ | ♀ | ♂ | ♀ | ♂ | ♀ |
| 26·3 | 35·0 | 41·8 | 47·0 | 35·4 | 48·2 |

In spite of the wide variation the differences in Table III are significant and indicate that the stimulation of cellular metabolism, whether caused directly by dinitrophenol or indirectly by the administration of thyroxine, does not accelerate the depletion of reserves of vitamin A in the liver but rather tends to conserve them.

Our results do not support the view that thyroxine increases the utilization or decreases storage of vitamin A [Euler & Klussmann, 1932; Fasold & Peters, 1933; Wendt, 1935].

It is too early to discuss the possible significance of this observation, but, meanwhile, it is of interest to note that Wolff [1932] and Moore [1937] have recorded that the liver reserves in human cases of hyperthyroidism are usually higher than the normal level.

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# CXXXI. THE ACTION OF CYSTINE AND METHIONINE ON LIVER FAT DEPOSITION

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Beeston & Channon [1936] reported early studies on the question as to whether the preventive action of proteins, such as caseinogen, on fat deposition in the liver was to be attributed to one or more of the constituent amino-acids. Investigation of the action of cystine showed that, so far from preventing fat deposition, this particular amino-acid accelerated it in a remarkable manner. Thus the mean percentage of fat in the livers of 45 control animals receiving a diet containing 40 % fat and 5 % caseinogen was increased from 21·5 to 31·78 by cystine, while the actual weight of fat in the liver of the 100 g. rat rose from 1·08 to 2·02 g., very marked effects being obtained with an intake of 5–7 mg. cystine per rat per day. Further, some extremely high values were encountered in individual animals receiving cystine, the highest being one in which the liver constituted 11·88 % of the body weight and contained 48·96 % of its fresh weight as fat (5·82 g. of fat in the liver of a 100 g. rat).

These results with cystine led naturally to a similar study of methionine which is reported in the present paper. While this work was in progress Tucker & Eckstein [1937] recorded results of studies of both cystine and methionine. Their results with cystine fully confirm those of Beeston & Channon [1936] referred to above. They further observed that methionine in contrast with cystine inhibited liver fat deposition and in consequence suggested that the lipotropic action of any protein depends on the balance between its contents of cystine and methionine. This suggestion is discussed later in this paper.

## Experimental

Exp. 1 was carried out in order to compare the actions of cystine and methionine on the "cholesterol" fatty liver. The control group of 9 male rats received the following diet: caseinogen (alcohol-extracted) 5, beef dripping 20, glucose 62, marmite 5, cod liver oil 1, salts 5, cholesterol 2. The two other groups of 7 and 9 animals received the same diet with the addition of 0·2 % cystine and 0·25 % methionine respectively, these two percentages being approximately equimolecular. After 21 days' feeding the animals were guillotined, and the total lipoids extracted from the pooled livers of each group by the alcohol-ether method. Free and total cholesterol, ether-soluble phosphorus, fatty acids and unsaponifiable matter were then determined on the total ethereal extract, and the composition of the liver lipoids calculated as previously described. The results are presented in Table I, from which the lecithin figures have been omitted, since neither in this nor the later experiments did they show changes of interest.

The striking effect of the administration of cystine is shown in Table I where the glyceride has increased from the control figure of 13·24 to 32·65 % of the fresh liver weight. These figures correspond to the presence in the liver of 0·569 g.

Table I. *Exp. 1. The effects of cystine and methionine in "cholesterol" fatty liver production*

| Group | Final body wt. g. | Wt. change ± % initial body wt. | Food intake g./rat/day | Liver % final body wt. |
|---|---|---|---|---|
| Control | 195 | − 5·5 | 8·5 | 4·3 |
| Control + methionine | 180 | −11·9 | 7·7 | 4·0 |
| Control + cystine | 216 | 0 | 9·0 | 6·3 |

| | Liver lipoids | | | | | |
|---|---|---|---|---|---|---|
| | % fresh liver wt. | | | g. in liver of 100 g. rat | | |
| Group | Cholesterol | Cholesteryl oleate | Glyceride | Cholesterol | Cholesteryl oleate | Glyceride |
| Control | 0·291 | 3·197 | 13·24 | 0·012 | 0·137 | 0·569 |
| Control + methionine | 0·289 | 2·212 | 10·12 | 0·012 | 0·088 | 0·441 |
| Control + cystine | 0·257 | 3·352 | 32·65 | 0·016 | 0·211 | 2·057 |

of glyceride in the control animals, and 2·057 g. in the livers of those receiving cystine. Thus the daily ingestion of only 18·2 mg. cystine per rat has increased the percentage of glyceride to two and a half times, and the absolute weight to nearly four times that of the control animals which is already 10 to 15 times greater than the normal amount. This experiment thus extends the previous finding of the accelerating effect of cystine on the deposition of glyceride in the "fat" fatty liver to that of the "cholesterol" fatty liver.

In clear contrast, methionine has had no effect in increasing the glyceride deposition. In the methionine group the glyceride percentage, 10·12, is actually 3·12 units lower than the control figure of 13·24 and the weight of glyceride in the liver 0·441 is also somewhat less than the 0·569 g. of the control group. While the results suggest that methionine might actually have had a slight effect in decreasing the liver fat, our previous experience of the variations which are to be met with even in groups of 10 animals are such as to make this deduction unwarranted. From this experiment we concluded, therefore, that a methionine intake of 19·25 mg. per rat per day had no effect, whereas a cystine intake of 18·2 mg. strikingly increased the amount of glyceride in the livers [Beeston *et al.* 1937].

The next experiment was carried out on animals receiving a diet calculated to produce "fat" fatty livers. Four groups of ten animals were used. The two control groups received the following diet: caseinogen (alcohol-extracted) 5, beef dripping 40, cod liver oil 1, glucose 49, inorganic salts 5, aneurin 10 $\mu$g. per rat per day. The two other groups received the same diets supplemented with 0·25 and 0·5 % of methionine respectively dissolved in the water used in the preparation of the diets. The animals received this diet for 16 days, and the unsaponifiable matter and fatty acids ("fat" of Table II) were extracted from each liver after saponification, firstly by aqueous, followed by alcoholic NaOH. The results are recorded in Table II as mean values, the extreme values being quoted in brackets.

Table II shows that the percentages of fat in the livers of the two control groups are 16·4 and 13·21, corresponding to 0·677 and 0·509 g. of fat respectively in the liver of the 100 g. rat. As previously pointed out, variations of this magnitude are to be expected in experiments of this type. Both the percentages of fat in the liver, 15·16 and 12·53, and the weight of fat in the liver of the 100 g. rat of the two groups of animals receiving methionine, 0·535 and 0·503 g., are in our

Table II. *Exp. 2. The effect of methionine in "fat" fatty liver production*

| Group | No. of animals | Initial body wt. g. | Wt. loss % initial body wt. | Liver % body wt. | Fat % liver | Fat in liver of 100 g. rat g. |
|---|---|---|---|---|---|---|
| Control 1 | 10 | 167 | −11·0 | 3·91 | 16·40 | 0·677 |
| Extremes | | | | (5·64–3·28) | (33·40–6·70) | (1·8860–0·2198) |
| Control 2 | 9 | 168 | − 9·4 | 3·86 | 13·21 | 0·509 |
| Extremes | | | | (4·39–3·34) | (17·71–9·23) | (0·7101–0·3493) |
| Methionine 0·25 % | 10 | 167 | − 6·2 | 4·06 | 15·16 | 0·535 |
| Extremes | | | | (4·89–3·45) | (27·63–12·66) | (1·4340–0·4220) |
| Methionine 0·5 % | 10 | 166 | − 5·8 | 3·96 | 12·53 | 0·503 |
| Extremes | | | | (5·28–3·15) | (22·62–6·33) | (1·0201–0·2597) |

experience not significantly different from those in the controls. The food intake in this experiment was not measured but in experiments on this diet by animals of this weight it is usually about 8 g. per rat per day. This would correspond to the intake of 20 and 40 mg. of methionine per rat per day in the two groups. The results of this experiment, therefore, are similar to those of Exp. 1 in showing that, in contrast to cystine, methionine at a considerably greater daily intake has had no effect on liver fat deposition. At this point the paper by Tucker & Eckstein [1937] appeared, in which, in contrast to the three results of Exps. 1 and 2, they recorded results showing that methionine had a marked action in decreasing fat deposition. Inspection of their data showed that the amount of "fat" in the livers of their control groups was 19–20 % as compared with 13–16 % of the three groups of Exps. 1 and 2 of the present work. This suggested an explanation of these divergent findings, for it is our general experience that the ease of prevention of fat deposition in the liver by a given substance depends on the amount which would have been present in the absence of that substance from the diet. The next two experiments were therefore carried out under conditions calculated to produce much more fatty livers, and for this purpose, acting on previous experience, albumin was substituted for caseinogen in order to increase the glyceride fraction. In Exp. 3, designed to produce the "fat" fatty liver, the control group of animals received a diet consisting of egg albumin 5, beef dripping 40, cod liver oil 1, glucose 49, salt mixture 5 parts. In Exp. 4 the control group received egg albumin 5, beef dripping 30, cod liver oil 1, cholesterol 2, glucose 57 and salt mixture 5 parts. The methionine groups in each experiment received the same diets with the addition of 0·5 % methionine. Further, all the animals received 10 $\mu$g. aneurin per day. In Exp. 3, the individual livers were worked up as described for Exp. 2, and in Exp. 4 the pooled livers were analysed as described for Exp. 1. The results are recorded in Tables III and IV.

Table III shows that the "fat" percentage of the livers of the methionine group, 14·06, is 9·55 units less than that of the control group, 23·61, with a corresponding decrease in the weight of fat from 1·460 to 0·6262 g. The glyceride fraction in the "cholesterol" fatty livers of Exp. 4 shows a considerable decrease

Table III. *Exp. 3. The effect of methionine in "fat" fatty liver prevention*

| Group | No. of animals | Initial body wt. g. | Wt. loss % | Liver % final body wt. | "Fat" % fresh liver | Fat in liver of 100 g. rat g. |
|---|---|---|---|---|---|---|
| Control | 9 | 169 | 5·4 | 5·54 | 23·61 | 1·460 |
| Extremes | | | | (3·81–8·27) | (10·68–40·05) | (0·4067–3·3120) |
| Control + methionine | 9 | 169 | 9·2 | 4·29 | 14·06 | 0·6262 |
| Extremes | | | | (3·57–5·03) | (7·03–18·9) | (0·2519–0·9500) |

Table IV. *Exp. 4. The effect of methionine in the prevention of glyceride deposition in "cholesterol" fatty livers*

| Group | No. of animals | Average initial body wt. g. | Wt. change ±% initial body wt. | Liver % final body wt. |
|---|---|---|---|---|
| Control | 10 | 172 | −8·9 | 6·89 |
| Control + methionine | 10 | 171 | −8·8 | 5·09 |

| | Liver lipoids | | | | | |
|---|---|---|---|---|---|---|
| | % fresh liver wt. | | | g. in liver of 100 g. rat | | |
| Group | Cholesterol | Cholesteryl oleate | Glyceride | Cholesterol | Cholesteryl oleate | Glyceride |
| Control | 0·262 | 1·673 | 33·78 | 0·018 | 0·194 | 2·327 |
| Control + methionine | 0·244 | 1·278 | 19·64 | 0·012 | 0·109 | 1·000 |

also, from 33·78 to 19·64 % and from 2·327 to 1·000 g. Both Exps. 3 and 4 therefore confirm the findings of Tucker & Eckstein that methionine has a preventive action on glyceride deposition in the liver when fed in the concentration used by them, 0·5 %, and when the glyceride percentage in the liver is sufficiently high. Evidence concerning the relative magnitudes of this action and that of choline was then sought in Exp. 5 on the "cholesterol" fatty liver.

In this experiment two groups of animals received the same diets as were used in Exp. 4, i.e. the "cholesterol" fatty liver-producing diet with 8 % albumin as the protein with and without 0·5 % methionine. A third group received the control diet with the addition of 0·04 % choline chloride. Two other groups were also used. The fourth group received the control diet with the substitution of 8 % caseinogen for the 8 % albumin, while the fifth received this caseinogen diet with the addition of 0·5 % methionine. A group receiving both caseinogen and choline chloride was not included, since we have already sufficient data concerning the action of choline on the "cholesterol" fatty liver when caseinogen is the protein of the basal diet. All the groups contained 10 animals

Table V. *Exp. 5. Preventive effect of methionine on the "cholesterol" fatty liver*

| Group | Average initial body wt. g. | Wt. change ±% initial body wt. | Liver % final body wt. |
|---|---|---|---|
| 8 % albumin | 169 | −3 | 7·80 |
| 8 % albumin + 0·04 % choline chloride | 167 | +1 | 5·86 |
| 8 % albumin + 0·5 % methionine | 164 | +3 | 5·09 |
| 8 % caseinogen | 164 | −7 | 6·63 |
| 8 % caseinogen + 0·5 % methionine | 164 | 0 | 4·53 |

| | Liver lipoids | | | | | |
|---|---|---|---|---|---|---|
| | % fresh liver wt. | | | g. in liver of 100 g. rat | | |
| Group | Cholesterol | Cholesteryl oleate | Glyceride | Cholesterol | Cholesteryl oleate | Glyceride |
| 8 % albumin | 0·31 | 4·55 | 36·20 | 0·024 | 0·355 | 2·82 |
| 8 % albumin + 0·04 % choline chloride | 0·32 | 5·47 | 27·14 | 0·019 | 0·321 | 1·59 |
| 8 % albumin + 0·5 % methionine | 0·29 | 3·97 | 21·30 | 0·015 | 0·200 | 1·08 |
| 8 % caseinogen | 0·34 | 4·98 | 32·19 | 0·023 | 0·330 | 2·13 |
| 8 % caseinogen + 0·5 % methionine | 0·30 | 3·44 | 17·79 | 0·014 | 0·156 | 0·81 |

which were fed for 21 days, when the pooled livers were completely analysed as in Exp. 1. The data are presented in Table V.

Confining attention for the moment to the glyceride fraction, it is seen that in both the control albumin and caseinogen groups very high figures resulted, 36·20 and 32·19 % and 2·82 and 2·13 g. respectively. The inclusion of 0·5 % methionine has reduced each of these control figures by some 15 units %, the decreases in weight being 1·8 and 1·3 g. respectively. These results show that methionine has had a greater effect on the livers of the albumin group than on those of the caseinogen group. This may be connected with the greater fat content of the livers of the former, but the nature of the protein may also be involved. The livers of the animals receiving 0·04 % choline chloride with 8 % albumin contain 27·14 % and 1·59 g. per 100 g. rat as compared with 36·20 and 2·82 g. of the albumin group. These reductions are about 60 % of that caused by 0·5 % methionine.

## Discussion

The results of Exps. 3–5, which are in effect four separate observations, show clearly that the inclusion of 0·5 % methionine in a diet will prevent glyceride deposition in the livers of rats receiving diets high in fat with or without added cholesterol. The results of these experiments in which the control diet has caused the appearance of more than 20 % glyceride in the liver, confirm those of Tucker & Eckstein [1937]. Those of Tables I and II, consisting of three experiments, show equally clearly the lack of effect of 0·25 % methionine in two experiments, and 0·5 % in a third, when the control glyceride percentage is below 16 %. From the present results and from much previous work on the action of choline, it is clear that the action of methionine is far less intense than is that of choline itself, and it is of value to attempt to deduce something of the magnitude of its action. In all the experiments in which an action has been observed the diet has contained 0·5 % methionine, corresponding to an intake of about 40 mg. per rat per day. It is this figure, therefore, which must be equated with choline. In the albumin groups of Exp. 5 a daily intake of about 3 mg. choline has had about 60 % of the action of this amount of methionine, but there still remains in the liver a very high percentage of glyceride, 21·3 %. It seems reasonable in the light of previous experience to believe that 4–6 mg. choline would have had an effect equal to that of 40 mg. methionine in this experiment. Comparison may also be made with the action of choline in experiments carried out for other purposes. In any such indirect comparison, it is necessary that the level of glyceride be approximately the same as that which occurred in the control groups of the methionine experiments, and in the present state of knowledge it appears desirable that only experiments in which the same protein was used be compared. There are accordingly recorded in Table VI unpublished data for comparison with those of Exps. 3 and 4 and the second part of Exp. 5.

The percentage and weight of fat in Exp. (*a*) of Table VI are very similar to those of Exp. 3, the 2·2 mg. choline having had a rather greater effect than the 40 mg. methionine in that experiment. Accepting the actions as identical, the effect of methionine is $\frac{1}{18}$ that of choline. In Exp. (*b*), Table VI, the figures are also very similar to those of Exp. 4, indicating methionine to have $\frac{1}{20}$ of the activity of choline. In Exp. (*c*), Table VI, 4·1 mg. choline have had an action similar to the 40 mg. methionine of the second part of Exp. 5. This experiment also shows the effect of increasing the choline intake to 9·2 mg., for the percentage and weight of glyceride fall well below that caused by methionine in Exp. 5. The results of the first part of Exp. 5 and of the three comparisons just recorded indicate the activity of methionine to lie between $\frac{1}{8}$ and $\frac{1}{20}$ that of

Table VI

| | Fat in liver % | Fat in liver of 100 g. rat g. | |
|---|---|---|---|
| "Fat" fatty liver | | | |
| (*a*) 8% albumin | 25·95 | 1·467 | cf. Exp. 3, Table III |
| 8% albumin + 2·2 mg. choline/rat/day | 13·30 | 0·532 | |
| | Glyceride in liver % | Glyceride in liver of 100 g. rat g. | |
| "Cholesterol" fatty liver | | | |
| (*b*) 8% albumin | 36·58 | 3·106 | cf. Exp. 4, Table IV |
| 8% albumin + 2 mg. choline/rat/day | 25·66 | 1·506 | |
| (*c*) 5% caseinogen | 31·30 | 1·960 | cf. Exp. 5, part 2, Table V |
| 5% caseinogen + 4·1 mg. choline/rat/day | 13·73 | 0·630 | |
| 5% caseinogen + 9·2 mg. choline/rat/day | 7·44 | 0·294 | |

choline. This low activity of methionine explains our failure to observe any effect of this amino-acid in Exps. 1 and 2.

The results of this work and of that of Beeston & Channon [1936] thus provide an interesting illustration of the opposing action of these two amino-acids, cystine in enormously accelerating fat deposition in the liver and methionine in exerting a preventive action of less striking character. These facts are of particular significance in view of the growing body of evidence indicating that cystine and methionine do not follow the common metabolic path assigned to them by Jackson & Block [1932] [cf. Brand *et al.* 1935; Lewis *et al.* 1936; Rose, 1937; Rose *et al.* 1937; White & Beach, 1937]. In this connexion the actions of S-methylcysteine and homocystine on liver fat are being investigated in this laboratory.

Since the original observations of Beeston & Channon [1936] on the accelerating action of cystine in fat deposition in the liver, it has been obvious that the action of proteins in preventing fat deposition—the so-called lipotropic action—must be the result of a balance between factors accelerating and preventing fat deposition, provided that amino-acids liberated from proteins during digestion behave in the same way as amino-acids added to a diet. The observations on methionine recorded here and those of Tucker & Eckstein [1937] provide the first example of an amino-acid decreasing liver fat. The latter authors have in fact made the suggestion that the lipotropic action of any protein depends on its relative cystine and methionine contents. In this connexion it is to be noted that the effect of the daily intake by the rat of 0·5 g. caseinogen can be readily seen in the decrease in the amount of fat in its liver. This amount of caseinogen would contain about 17 mg. methionine, which on the evidence presented in this paper would be equivalent in its action to, say 1·5 mg. choline. While amounts of choline of this order may have a marked action depending on the percentage of fat which would occur in the liver in their absence from the diet and possibly on the nature of the dietary protein, it seems to us desirable to await considerable further evidence before a judgment can be made as to whether the methionine and cystine contents of proteins account entirely for their lipotropic action. The completion of our studies on the effect of the different amino-acids, and work which is in progress in which pure proteins of different cystine and methionine contents are being supplemented with these amino-acids in order to compare them from this point of view, will provide such evidence. Further, it has yet to be established that the lipotropic action of

proteins is due to one or more constituent parts of the molecule, and not to contaminants.

Attention may be drawn to the fact that the administration of methionine has decreased the amount of cholesteryl ester present in the liver of the 100 g. rat in all the experiments in which cholesterol was employed, i.e. Exps. 1 and 4, and both parts of Exp. 5, the decreases in Exps. 4 and 5 being of the order of 50% of the control value. The decrease in Exp. 1, in which the amount of cholesteryl oleate, 0·088 g., is little more than 60% of the control value, 0·137 g., is particularly interesting since methionine caused no decrease in the glyceride fraction in this experiment. In all these experiments, save Exp. 1, there has also been a decrease in the amount of free cholesterol in the livers. These decreases in cholesterol and cholesteryl esters also occur when choline is administered, although we have not observed in a large range of experiments an effect on the cholesterol fractions without an effect on the glyceride fractions.

## Summary

1. A study has been made of the effect of the administration of methionine to rats receiving low protein diets of high fat content with and without added cholesterol, on lipoid deposition in the liver.

2. In two experiments on the "fat" fatty liver, an intake of 20 and 40 mg. methionine per rat per day had no preventive effect when the "fat" in the livers of the control animals was 16·4 and 13·2%. In a third experiment 40 mg. had a marked preventive action when the fat in the liver was 23·6%.

3. Similar results were obtained in experiments on the "cholesterol" fatty liver. In one experiment 20 mg. methionine had no effect when the glyceride percentage in the control groups was 13·2%. In three further experiments, in which the control glyceride percentage was greater than 20, 40 mg. methionine had a considerable preventive action.

4. Evidence is adduced that the intensity of action of methionine in preventing glyceride deposition in the liver is about $\frac{1}{12}$ that of choline.

5. The striking effect of the administration of small amounts of cystine in increasing the deposition of glyceride in "cholesterol" fatty livers is demonstrated.

6. The administration of methionine with cholesterol-containing diets markedly decreases the deposition of cholesterol, both free and as ester.

7. The bearing of these findings of the antagonistic actions of methionine and cystine on the problem of metabolism of these amino-acids and on the possibility that the lipotropic action of proteins may be accounted for by this action is discussed.

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# CXXXII. THE EFFECT OF PROTEINS IN THE PREVENTION OF DIETARY FATTY LIVERS

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Rats fed on low protein diets of high fat content with or without small amounts of cholesterol develop intensely fatty livers; when caseinogen is used as the dietary protein, the degree of fat infiltration diminishes on increasing the amount of this protein present in the diet, irrespective of any action of choline [Channon & Wilkinson, 1935; Beeston *et al.* 1935]. Further work has shown that a daily intake of 1 g. caseinogen has an effect equivalent to that of 7–8 mg. choline [Beeston & Channon, 1935; Beeston *et al.* 1936]. Best *et al.* [1936] obtained similar results regarding this extensive action of caseinogen on liver fat deposition in preventive experiments. While we are continuing our studies of the action of pure amino-acids as one method of elucidating the mechanism of this action of caseinogen [Beeston & Channon, 1936; Channon *et al.* 1938], information as to the relative lipotropic actions of other proteins is desirable for two reasons. Firstly, it would enable the intensity of action of different proteins to be studied in relation to their amino-acid composition, and perhaps provide evidence of value from this point of view; secondly, it is important to determine which proteins are most satisfactory from the experimental point of view. Diets low in protein tend to cause weight losses even in the short term studies often used in this type of work, and a balance has to be struck between the desirable high fat percentage in the liver and ensuing weight losses. The work described in this paper is, therefore, a study of the actions of a number of plant and animal proteins in preventing fat deposition in the liver, caseinogen being used as the standard.

## Experimental

### *Preparation of the proteins*

*Edestin* was crystallized from 5% NaCl extracts of hempseed, washed twice with water, twice with alcohol and with ether; the dried powder was extracted twice with hot alcohol and with ether. Yield 75–80 g./kg. hempseed.

*Fibrin* obtained from the slaughter-house was water-washed until colourless, and dried in alcohol; the powdered product was extracted with water, cold alcohol, hot alcohol and finally with ether.

*Gliadin.* Repeated extraction with 73% alcohol (3, 3 and 2 l.) of wheat gluten flour (1 kg.), concentration *in vacuo* of the extracts and cooling in the refrigerator gave a jelly of gliadin, which was dehydrated by successive treatments with acetone; the finely ground product was extracted with cold absolute alcohol and with ether. Yield 275–290 g./kg. gluten flour.

*Zein.* Maize gluten meal (1 kg.) was extracted repeatedly with hot 80% alcohol (4, 2 and 2 l.), the extract concentrated to 2·5 l., and mixed with 2·5 l. of ether. The gelatinous precipitate was dissolved in warm 90% alcohol and

allowed to drip slowly from a separating funnel into running water; the precipitated zein was collected, washed in water, air-dried and purified by extraction with ether. Yield 130–150 g./kg. maize gluten meal.

*Beef muscle protein.* Lean meat from which fat had been carefully dissected was minced, dried in alcohol and ground; the powder was repeatedly extracted with hot alcohol and hot ether.

*Gelatin.* "Cox's Instant Powdered Gelatin" was extracted twice with alcohol and once with ether.

*Whale muscle protein and Gromax*, commercial preparations of whale meat kindly supplied by Messrs Lever Bros. and Unilever, Ltd., were purified by repeated extraction of the crude material with boiling alcohol and with ether; after the first few extractions the product was ground to a fine powder.

*Albumin* was a commercial preparation from eggs. *Caseinogen* was an alcohol-extracted preparation supplied by Glaxo, Ltd. Neither of these materials was further extracted since they were found to be essentially choline-free.

Nitrogen, moisture and ash determinations were made on each of the materials prepared. The latter was approximately 4 % for the beef and whale muscle proteins, 26 % for the Gromax, 1·5 % for the gelatin and less than 0·5 % for the other proteins.

Choline was estimated in each of the products by hydrolysis, acetylation and assay on isolated rabbit intestine; the whale meat protein contained 34 mg. and 53 mg. choline/100 g. in the two batches, the Gromax 18 mg./100 g., but in all the other proteins used the choline content was less than 9 mg./100 g. and in many cases less than 4 mg./100 g.

*The animal experiments*

In Exp. 1 the control group of rats received a diet which would cause "cholesterol" fatty livers, consisting of caseinogen (alcohol-extracted) 5, beef dripping 30, cod liver oil 1, cholesterol 2, marmite 5, salts 5 and glucose 52 parts. Seven other groups received a similar diet save that 10 parts of different proteins were substituted for 10 parts glucose. There were 9 or 10 female rats in each group, and the period of feeding was 21 days. At the end of the experiment, the livers were pooled, and the fatty acids + unsaponifiable matter prepared by the saponification method. The results are recorded in Table I, in which "fat" in the heading of the last column but one refers to the sum of the fatty acids and unsaponifiable matter: it will, on this particular diet, contain about 3 parts of cholesterol but this will vary little from group to group. The difference between the percentages and amounts of "fat" of the control group and of the other groups will, therefore, to all intents and purposes represent a change in the glyceride content of the liver caused by the inclusion in the diet of 10 % of each particular protein.

In Exp. 2 the control group of animals received a diet consisting of caseinogen (alcohol-extracted) 8, beef dripping 39, cod liver oil 1, glucose 47, salt mixture 5 parts; in addition each animal received 12·5 $\mu$g. aneurin and 25 $\mu$g. lactoflavin per day. Ten other groups received the same diet, save that 7 parts of different proteins were substituted for 7 parts of glucose. All the groups contained 10 rats, and were fed for 21 days. The animals were killed by guillotine and the individual livers worked up by the saponification method for fatty acids and unsaponifiable matter. The results are recorded in Table II as mean values for the ten individual rats of each group, and hence figures in the last column for the weight of fat in the liver of the 100 g. rat may differ slightly from the product of the two previous columns which record the liver as a percentage of the

final body weight and the percentage of fat in the fresh liver. The term "fat" means the sum of the fatty acids and the unsaponifiable matter, as it does in all the experiments.

In Exps. 3 and 4 (Table III, A and B) the control diet was the same as in Exp. 2 save that the basal protein was egg albumin instead of caseinogen, while the lactoflavin was omitted. In Exp. 3 the period of feeding was 17 days, and in Exp. 4, 18 days. The livers were investigated in Exps. 3–6 as described for Exp. 2. Details of the diets used in Exps. 5 and 6 are recorded later in the text.

## Results

In Exp. 1 the proteins were used as 10% supplements, since in the work of Beeston *et al.* [1935] 10% of caseinogen had proved very effective on the "cholesterol" fatty liver when supplementing a basal 5% of the same protein.

Table I. *Exp. 1. The effect of protein supplements on lipoid deposition in the liver of rats receiving a diet containing fat and cholesterol*

| Group | Protein of diet | No. of animals | Final body wt. g. | Change in wt. ± % initial body wt. | Liver % final body wt. | "Fat" % fresh liver | "Fat" in liver of 100 g. rat |
|---|---|---|---|---|---|---|---|
| 1 | 5% caseinogen | 10 | 149 | −4·6 | 5·22 | 32·55 | 1·699 |
| 2 | 5% caseinogen + 10% caseinogen | 10 | 164 | +3·7 | 4·52 | 22·14 | 1·001 |
| 3 | 5% caseinogen + 10% edestin | 10 | 168 | +5·7 | 4·73 | 23·76 | 1·124 |
| 4 | 5% caseinogen + 10% fibrin | 10 | 165 | +6·7 | 5·14 | 27·63 | 1·420 |
| 5 | 5% caseinogen + 10% gelatin | 9 | 157 | −2·1 | 5·54 | 35·07 | 1·943 |
| 6 | 5% caseinogen + 10% gliadin | 10 | 171 | +9·1 | 5·15 | 30·33 | 1·562 |
| 7 | 5% caseinogen + 10% Gromax | 10 | 164 | +4·1 | 4·37 | 27·12 | 1·185 |
| 8 | 5% caseinogen + 10% zein | 9 | 145 | −4·2 | 4·75 | 27·15 | 1·290 |

Table I shows that the addition of 10% of caseinogen has lowered the liver-fat percentage from 32·55 in the control group to 22·14, and decreased the weight of fat in the liver of the 100 g. rat by 0·698 g. The effect of this percentage of caseinogen, therefore, while not so great as in the work quoted above, is sufficiently pronounced for deductions to be made concerning the relative actions of the other protein supplements. It should be pointed out that, in this paper, more weight has been attached to the values for liver fat/100 g. rat than to the percentages of fat in the fresh liver, though these latter figures have, of course, been considered. Edestin and Gromax have had actions of approximately equal intensity to that of caseinogen; in a second group zein and fibrin may be classed together; gliadin appears to have had little if any effect, while gelatin has caused an increase in the amount of fat to 1·943 g. which is about 15% greater than the control value.

In Exp. 2 the observations of Exp. 1 were extended to a study of the "fat" fatty liver, and the amount of caseinogen, the basal protein of the diet, was increased to 8% in order to avoid as far as possible weight losses, which tend to occur on diets containing 5% caseinogen and 40% fat. At the same time, egg albumin and the proteins of whale and beef muscle were also investigated, all the proteins being fed as 7% supplements.

The percentage and weight of fat in the control group, 27·12 and 1·664 g., are both very satisfactory from the point of view of studying the comparative effects of the different proteins. The addition of 7% caseinogen has lowered the percentage of fat to 17·92 and decreased the weight per 100 g. rat to 0·843 g., which is more than half of the control value. The results in Table II place the

Table II. *Exp. 2. The effects of protein supplements on fat deposition in "fat" fatty livers*

| Group | Protein in diet | Final body wt. g. | Change in wt. ±% initial body wt. | Liver % final body wt. | Fat % fresh liver | Fat in liver of 100 g. rat g. |
|---|---|---|---|---|---|---|
| 1 (Control) | Caseinogen 8 | 180 | + 3 | 5·99 | 27·12 | 1·664 |
| 2 | Caseinogen 8 + albumin 7 | 200 | +11 | 4·11 | 19·73 | 0·880 |
| 3 | Caseinogen 8 + beef muscle 7 | 197 | + 7 | 4·49 | 20·31 | 0·994 |
| 4 | Caseinogen 8 + caseinogen 7 | 200 | +10 | 4·28 | 17·92 | 0·843 |
| 5 | Caseinogen 8 + edestin 7 | 197 | + 8 | 4·80 | 20·82 | 1·078 |
| 6 | Caseinogen 8 + fibrin 7 | 202 | +13 | 4·40 | 19·70 | 0·906 |
| 7 | Caseinogen 8 + gelatin 7 | 192 | + 5 | 4·31 | 24·70 | 1·094 |
| 8 | Caseinogen 8 + gliadin 7 | 188 | + 2 | 4·53 | 21·29 | 1·007 |
| 9 | Caseinogen 8 + Gromax 7 | 188 | + 1 | 3·86 | 17·71 | 0·738 |
| 10 | Caseinogen 8 + whale muscle 7 | 201 | + 8 | 3·59 | 14·57 | 0·570 |
| 11 | Caseinogen 8 + zein 7 | 184 | + 1 | 4·07 | 18·53 | 0·801 |

protein supplements in three classes as regards the order of their effect in preventing fat deposition:

(*a*) proteins having an intensity of action similar to that of caseinogen—albumin, fibrin, Gromax and zein;

(*b*) proteins, the effect of which is greater than that of caseinogen—proteins of whale muscle;

(*c*) proteins having a less intense action than that of caseinogen—beef muscle protein, edestin, gelatin and gliadin.

It is clear from Tables I and II that certain of the proteins have not given consistent results in these two experiments, and this matter will be discussed later.

As a result of Exps. 1 and 2 it was felt that this method of experimentation in which supplements of 7 or 10 % protein were used would not provide adequate evidence concerning proteins of low lipotropic activity. It seemed, therefore, desirable to carry out Exps. 3 and 4 in which the proteins were added as 20 % supplements. The greater effect which should be observable with these larger supplements would serve the further purpose of eliminating the uncertainty

Table III. *The effect of protein supplements in decreasing liver fat*

| Group | Protein in diet | No. of animals | Final body wt. g. | Change in wt. % initial body wt. | Liver % final body wt. | Fat % fresh liver | Liver fat per 100 g. rat g. |
|---|---|---|---|---|---|---|---|
| | | A. Exp. 3 | | | | | |
| Control | 8% albumin | 9 | 177 | − 4 | 5·56 | 28·84 | 1·652 |
| 2 | 8% albumin + 20% albumin | 10 | 200 | +10 | 3·75 | 7·87 | 0·301 |
| 3 | 8% albumin + 6% caseinogen | 10 | 200 | + 6 | 5·20 | 24·94 | 1·382 |
| 4 | 8% albumin + 13% caseinogen | 9 | 200 | +11 | 3·91 | 12·87 | 0·524 |
| 5 | 8% albumin + 20% caseinogen | 10 | 206 | +11 | 3·58 | 8·29 | 0·289 |
| 6 | 8% albumin + 20% edestin | 10 | 202 | + 7 | 3·35 | 9·06 | 0·332 |
| 7 | 8% albumin + 20% gelatin | 10 | 173 | − 4 | 4·94 | 22·29 | 1·160 |
| 8 | 8% albumin + 20% gliadin | 9 | 206 | + 8 | 3·69 | 12·51 | 0·463 |
| 9 | 8% albumin + 20% Gromax | 10 | 189 | + 3 | 3·40 | 12·19 | 0·430 |
| 10 | 8% albumin + 20% zein | 10 | 192 | + 1 | 4·50 | 22·55 | 1·124 |
| | | B. Exp. 4 | | | | | |
| Control | 8% albumin | 10 | 166 | − 2 | 5·18 | 26·17 | 1·494 |
| 2 | 8% albumin + 20% beef muscle | 10 | 196 | +17 | 4·04 | 8·53 | 0·353 |
| 3 | 8% albumin + 20% fibrin | 9 | 179 | + 4 | 4·49 | 12·41 | 0·573 |
| 4 | 8% albumin + 20% whale muscle | 10 | 192 | +11 | 3·79 | 7·18 | 0·275 |

caused by variations in the different groups, due to unknown factors which cannot be controlled. In Exps. 3 and 4, 8% albumin was used instead of caseinogen, because it had been our general experience, as discussed later, that at low levels such as this albumin caused more fatty livers than did caseinogen.

The results of Exp. 3 show very clearly the successive decreases in the amount of liver fat caused by increasing the caseinogen content of the diet, for the figures of the control group, 28·84% and 1·652 g., of liver fat are progressively decreased until, when the diet is supplemented by 20% caseinogen, there remains in the liver only 8·29% and 0·289 g. This series of figures is typical of those obtained in a large number of similar experiments. Exp. 3 also shows that in the conditions used albumin and edestin have actions equal to that of caseinogen; in our experience the slightly higher figures which are obtained with these two proteins, 0·301 and 0·332, as compared with 0·289 g. with caseinogen, are not to be interpreted as significantly different. The results with gliadin, however, in which the liver fat is 12·51% and 0·463 g., show that it is less effective than caseinogen, edestin and albumin. The results show clearly also that both zein and gelatin have positive, though considerably slighter actions, for the amounts of fat in the liver with these two proteins, 1·160 and 1·124 g. respectively, are some 30% less than that of the control group 1·652 g., there being a fall both in the liver size and in the fat percentage in the livers. The interesting point that supplements of these two deficient proteins have an action on liver fat is elaborated later. In Exp. 4 the control figures 26·17% and 1·494 are very similar to those of Exp. 3. The results of this experiment show that the whale muscle protein is the most effective of the three proteins used, its action being as great as those of caseinogen, edestin and albumin in Exp. 3; beef muscle protein has an action which may not be significantly less, while the action of fibrin is definitely less intense. Although it might have been preferable to have included in Exp. 3 a further group with a supplement of caseinogen, the similarity of the control figures in Exps. 3 and 4 makes it reasonable to regard these two experiments as being essentially one. If this be done, the different protein supplements can be arranged in three groups as regards the intensity of their actions in preventing fat deposition in the liver, (*a*) the most active—albumin, caseinogen, edestin, the proteins of whale muscle and possibly of beef muscle, (*b*) proteins with a marked action but less than those of group (*a*)—gliadin, Gromax and fibrin and (*c*) proteins with a definite though considerably less intense action—gelatin and zein.

The finding that albumin had an effect as great as that of caseinogen was surprising in view of our previous experience in which this protein at low levels caused more fatty livers than caseinogen. Thus, in an early experiment carried out by Dr A. W. Beeston, a comparison was made of the effects of 5% albumin and of 5% caseinogen in diets containing 40% fat. The percentages of "fat" in the fresh livers and the actual weights in the liver of the 100 g. rat were: caseinogen 26·34%, 1·58 g.; albumin 33·77%, 2·28 g. At the same low level, therefore, the amount of fat in the liver is some 50% greater when albumin is the protein. Two further experiments, one on the "fat" and the other on the "cholesterol" fatty liver, gave results of the same order.

| "Cholesterol" fatty liver | | | "Fat" fatty liver | | |
|---|---|---|---|---|---|
| Protein in diet | Glyceride in liver % | Glyceride in liver of 100 g. rat | Protein in diet | Fat in liver % | Fat in liver of 100 g. rat g. |
| 8% caseinogen | 32·19 | 2·13 | 5% caseinogen | 21·30 | 1·077 |
| 8% albumin | 36·20 | 2·82 | 5% albumin | 29·52 | 1·712 |

The results of these three experiments in which the use of albumin in place of caseinogen resulted in the deposition in the liver of 30–70 % more fat, led us to compare the actions of these two proteins at a series of levels. The usual 40 % fat diet was used for 17 days, when the individual livers were worked up by the saponification method, the mean results being recorded in Table IV.

Table IV. *Exp. 5. Fat contents of the livers of rats receiving a diet containing 40 % fat with varying percentages of albumin or caseinogen*

| Group | Protein % of diet | No. of animals | Average initial body wt. g. | Change in wt. ± % initial wt. | Liver % final body wt. | Fat in liver % | Fat in liver of 100 g. rat g. |
|---|---|---|---|---|---|---|---|
| 1A | 8 % caseinogen | 9 | 183 | −5·6 | 4·75 | 20·51 | 1·000 |
| 1B | 8 % albumin | 10 | 179 | +3·3 | 5·86 | 29·10 | 1·758 |
| 2A | 14 % caseinogen | 9 | 178 | −0·1 | 4·87 | 22·56 | 1·099 |
| 2B | 14 % albumin | 10 | 182 | +6·2 | 6·19 | 24·64 | 1·564 |
| 3A | 21 % caseinogen | 10 | 182 | +2·8 | 4·42 | 8·62 | 0·390 |
| 3B | 21 % albumin | 8 | 175 | +5·1 | 4·91 | 10·28 | 0·505 |

The results in Table IV present an anomaly in that the amount of fat in the livers of the rats receiving 14 % caseinogen, 1·099 g., is slightly greater than that of those receiving 8 % caseinogen, 1·000 g. This is the only example of such an occurrence which we have encountered in a very large series, for the effect of increasing the caseinogen content of the diet by 5 % has invariably resulted in a fall in the amount of liver fat, a typical series being recorded in Table III. This happening, moreover, does not affect the interpretation of the results in Table IV. These, together with those already recorded, show that at all protein levels of 14 % or less, albumin produces considerably more fatty livers than does caseinogen, but that when its concentration is increased above this it manifests a very marked action on liver fat. Thus, whereas an increase in the albumin content of the diet from 8 to 14 %, an increased intake by the animal of about 0·5 g. per day, has lowered the weight of fat from 1·758 to 1·564 g., a decrease of only 0·194 g., a further increase to 21 % has caused it to decrease from 1·564 to 0·505 g.

A study was next made in more detail of the action of gelatin, since this protein is deficient in certain amino-acids. Four groups of animals received for 17 days a diet consisting of albumin 8, beef dripping 40, cod liver oil 1, salt mixture 5: the control group received 46 parts of glucose and in groups 2, 3 and 4 supplements of 6, 13 and 20 % of gelatin were added with a corresponding decrease in the glucose. All the diets contained aneurin such that each rat received 10 $\mu$g. per day. The livers were worked up individually for fatty acids and unsaponifiable matter by the saponification method. The mean results are recorded in Table V.

Table V. *Exp. 6. The effect of gelatin on the deposition of liver fat*

| Group | Protein in diet | No. of rats | Initial body wt. g. | % change in body wt. | Liver % final body wt. | Fat % fresh liver | Fat in liver of 100 g. rat g. |
|---|---|---|---|---|---|---|---|
| 1 | 8 % albumin | 10 | 183 | +1 | 5·5 | 27·56 | 1·642 |
| 2 | 8 % albumin + gelatin 6 % | 10 | 180 | +1 | 5·3 | 28·44 | 1·555 |
| 3 | 8 % albumin + gelatin 13 % | 10 | 182 | +8·2 | 5·2 | 25·83 | 1·402 |
| 4 | 8 % albumin + gelatin 20 % | 9 | 178 | −2·7 | 5·3 | 22·10 | 1·215 |

Table V confirms the result of Exp. 3 in that the addition of 6, 13 and 20% gelatin has caused a progressive decrease in the percentage of fat in the liver from 27·56 to 22·10, and in the absolute weight from 1·642 to 1·215 g., and with these confirmatory results there seems to be no doubt that gelatin supplements exert a definite but limited action in decreasing liver fat.

## Discussion

The results of the experiments described above are summarized in Table VI, in which the decreases of liver fat in the 100 g. rat caused by the protein supplements are expressed as percentages of the corresponding decrease due to an equal supplement of caseinogen.

Table VI. *Comparative effects of different proteins in decreasing liver fat*

| Exp. ... ... ... | 1 | 2 | 3 and 4 | 5 and 6 | |
|---|---|---|---|---|---|
| Protein basal % ... ... | Caseinogen 5 | Caseinogen 8 | Albumin 8 | Albumin 8 | Albumin 8 |
| Protein supplement % ... | 10 | 7 | 20 | 6 | 13 |
| Albumin | — | 95 | 99 | 40 | 102 |
| Beef muscle | — | 82 | 96 | — | — |
| Caseinogen | 100 | 100 | 100 | 100 | 100 |
| Edestin | 82 | 71 | 97 | — | — |
| Fibrin | 40 | 92 | 77 | — | — |
| Gelatin | −35 | 69 | 36 | 43 | 24 |
| | | | 34 (Exp. 6) | | |
| Gliadin | 20 | 81 | 87 | — | — |
| Gromax | 74 | 113 | 90 | — | — |
| Whale muscle | — | 133 | 102 | — | — |
| Zein | 59 | 105 | 39 | — | — |

Although Exp. 1 differed from the others in that the diet produced a "cholesterol" fatty liver, it is considered justifiable to include its results in this table and to compare them with those of the "fat" fatty liver experiments, since all the factors known to influence fat deposition exert an influence on both types of fatty liver.

While the results in Table VI are consistent with respect to some of the protein supplements, in other cases the lipotropic activity appears to vary with the nature of the basal protein. Thus beef muscle protein and edestin appear to be more effective, whale muscle protein, zein and perhaps gelatin less effective, when fed with albumin than when added to caseinogen. Further the amount of basal protein also appears to be of importance since fibrin, gelatin, gliadin, Gromax and zein all show conspicuously greater actions when fed as 7% supplements to 8% caseinogen in Exp. 2 than when fed as 10% supplements to 5% caseinogen in Exp. 1. The possible significance of these variations is mentioned below. No comment is offered on the increase in liver fat to a value greater than that of the control caused by gelatin in Exp. 1, shown as a negative value in Table VI. Deviations of this type in groups of animals have been encountered before, and it is not possible to decide whether this result is fortuitous or otherwise; the results of Exps. 2–6 suggest the former explanation.

Comment on the actions of two proteins in particular is necessary. Albumin when fed either as a supplement to caseinogen or alone is equal to that protein in activity at a level of 21 or 28%. The considerable data presented earlier regarding its action at 5 or 8% show that it invariably produces more fatty livers than caseinogen at these lower percentages. One result of its action at an intermediate value, 14%, occurs in Exp. 5, where the addition of 6% albumin

to a basal 8 % albumin has had little, if any, effect on the fat deposition. This result may be suspect because of the caseinogen value in the same experiment, but we think it probable that the figure is reliable. The fact that albumin has an activity less than that of caseinogen at 5 or 8 %, but equal to it at 20 % or more, may mean that its action is really less than that of caseinogen throughout, and that this fact is only observable at the lower levels where considerable differences occur. Alternatively, if the result with 14 % albumin in Exp. 5 is correct, it implies that there is a sudden increase in the intensity of its lipotropic action at higher levels, a fact which may be connected with the amino-acid make-up of the protein. Regarding this protein no published data are available, although Best *et al.* [1936] reported that unextracted egg white had an action similar to that of caseinogen.

Of the two deficient proteins studied the series of results in Exp. 6 shows that gelatin fed as a supplement has a definite though considerably less marked action, for the result of the 20 % group in Exp. 6 confirms that of Exp. 3, and the intermediate values at 6 and 13 % in Exp. 6 provide further evidence. The published figures of an experiment by Best *et al.* [1936] led them to suggest that gelatin had little if any action.

In considering all the data we feel that greater reliance should be placed on the results of Exps. 3 and 4 in which the added proteins constituted 20 % of the diet, since the higher percentage will emphasize differences in their action, and the more fatty livers resulting from the use of albumin as the basal protein will increase the sensitivity. It may be concluded that all the proteins are active to a greater or lesser extent irrespective of whether they supplement albumin or caseinogen. Lipotropic activity is most marked in the cases of Gromax and whale muscle protein; and if the lesser activity of Gromax be correlated with its higher ash content (26 %) it becomes probable that these two whale meat preparations are practically equal in this respect. In view of the fact, recorded earlier, that these two proteins contained appreciable quantities of choline, it must be pointed out that even in Exps. 3 and 4, where they were fed in greater amounts the daily intake of choline from this source did not exceed 1 mg./rat. From evidence accumulated in these laboratories it is considered very improbable that this amount of choline is responsible for any serious proportion of the activity found for either the Gromax or the whale muscle protein. These proteins then may be regarded as having actions equal to, if not exceeding, that of caseinogen. Beef muscle protein and edestin appear to have activities very nearly as great as that of caseinogen although Best *et al.* [1936] report the activity of beef muscle protein, prepared by essentially the same method, to be considerably less. Beeston *et al.* [1936] showed that edestin was active when fed alone but did not compare its action with that of caseinogen. Definitely less active are fibrin and gliadin, while the least activity is shown by gelatin and zein.

It is appropriate at this point to emphasize that the albumin and gelatin used were commercial products, and though it would, unquestionably, have been preferable to have used pure preparations, neither such materials nor facilities for their preparation were available to us.

It is possible that the lipotropic action of proteins is due to the action of their constituent amino-acids, since the administration of cystine and methionine in diets has been found respectively to increase and decrease liver fat [Beeston & Channon, 1936; Tucker & Eckstein, 1937; Channon *et al.* 1938]. If the lipotropic action of a protein is thus the algebraic sum of factors tending to increase or to decrease liver fat, then clearly both the amount and nature of the basal protein will exert a profound influence on the behaviour of supplements of

any other protein. Such considerations might account for the differences pointed out between the results of Exp. 1 and those of Exp. 2, and for the contrasts between Exps. 1 and 2 in which caseinogen was the basal protein, and those of Exps. 3, 4, 5 and 6 in which albumin was the basal protein. If the action of one or more factors does not increase beyond a certain limit, as the results of Beeston & Channon [1936] with cystine imply, then the effectiveness of protein supplements will not necessarily increase regularly on increasing the amount of the supplement; the results with low and high percentages of albumin are explicable on this basis. Exps. 3 and 4 where the total protein intake was 28% of the diet, provide the further conclusion that if the lipotropic factors are amino-acids, one or more of them must be essential amino-acids; if this were not so all diets of high protein content should be of the same activity, the lipotropic substance being synthesized by the animal. It must be borne in mind, however, that the apparent activities of gelatin and zein could also be due to provision of a considerably greater nitrogen intake. On diets low in protein the substances responsible for the lipotropic action may be preferentially used for other purposes, and only become available for such action when the nitrogen intake is considerably increased. The necessity, for instance, for providing ample nitrogen in diets when testing the essential nature of amino-acids has been stressed by Rose [1937].

Lastly, from the point of view of further studies on the fatty liver problem, these results show that commercial egg albumin at low levels causes very fatty livers without any weight loss, and that in order to provide more protein nitrogen it is possible to add gelatin, for a reasonable proportion of this protein will not significantly decrease the liver fat. Maclean *et al.* [1937] and McHenry [1937] have used beef muscle protein in liver fat studies since this protein gives a better growth response in young animals, and according to Best *et al.* [1936] has a smaller lipotropic action than caseinogen.

## Summary

1. The lipotropic activities of various choline-free protein preparations have been compared with that of caseinogen, by feeding them both as sole dietary protein and as supplements to albumin and to caseinogen.

2. All the proteins investigated have apparent lipotropic actions, in order of decreasing intensity—Gromax and whale muscle protein, caseinogen, albumin, beef muscle protein and edestin, fibrin and gliadin and, lastly, gelatin and zein.

3. Albumin has been investigated in greater detail, and though of an activity similar to that of caseinogen at high levels, it consistently gives more fatty livers when fed at low levels.

4. The influence of protein supplements on liver fat appears to depend on the amount and nature of the basal protein.

5. Suggestions are put forward as to the possibility of the lipotropic activity of proteins being due to constituent amino-acids.

6. The possibility of avoiding weight losses in fatty liver studies by using diets containing low proportions of albumin supplemented with gelatin is pointed out.

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# CXXXIII. THE CONSTITUTION OF CERTAIN NUTSHELLS

## II. THE PERICARP OF *CORYLUS AVELLANA* (FILBERT, COB, BARCELONA OR HAZEL NUT)

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The postfertilization development of the ovary of the hazel flower results in the formation from the outer integument of the ovule of a thin fibrous seed coat closely adherent to the embryo and from the ovary wall of a hard, lignified pericarp which is quite free from the seed coat at the time when the fruit is ripe. The work recorded in this paper was done on the pericarp of the hazel fruit. The results obtained are therefore more comparable with those of Emley [1928] for the hull of the peanut, *Arachis hypogea*, than with those for the shell of the Brazil nut, *Bertholletia excelsa* [Boswell, 1936], as the hull of the former is the pericarp of the fruit, the seed coat being a thin fibrous membrane closely adherent to the kernel, while the shell of the latter is the seed coat.

### Experimental

*Preparation of the fractions.* The shells, freed from all traces of kernel, were ground to a very fine powder in order to facilitate the separation of the cellulose from the ligno-cellulose complex. The benzene-, alcohol- and water-soluble extracts and the hemicelluloses A, A 1 and B were prepared by methods described previously [Boswell, 1936]. The lignin was prepared by treating the residue of ligno-cellulose insoluble in cold 4 % NaOH with 5 % $H_2SO_4$ at 100° for 3 hr. and afterwards with 72 % $H_2SO_4$ in the cold for 18 hr., followed by dilution to *c.* 3 % $H_2SO_4$ and heating for 5 hr. at 100°. The residue, washed free from acid and dried by increasing concentrations of alcohol and finally by ether, was regarded as lignin. The cellulose was isolated from another portion of the ligno-cellulose residue by the following method. The residue was treated with neutral solutions of NaOCl and $Na_2SO_3$ [Norman & Jenkins, 1933]. Three treatments removed all the easily soluble lignin leaving a light brown powder. Substitution of acid hypochlorite (5 ml. NaOCl with about 3 % available Cl and 2 ml. 20 % $H_2SO_4$) for the neutral solution removed the residual lignin only very slowly, 21·0 % of lignin still remaining insoluble after several treatments. When however gaseous Cl was used, each chlorination of 5 min. being followed by boiling with 3 % $Na_2SO_3$ [Sieber & Walter, 1913], three treatments sufficed to prepare the cellulose as a grey powder giving no red coloration with $Na_2SO_3$ after exposure to Cl. This difference in the response to Cl is of interest in view of the effects obtained by Norman & Shrikhande [1935] and their statement that "the results obtained after chlorination in solution were almost identical with those for gaseous chlorination". It may however be noted from the figures given by these workers that treatment of straw by gaseous Cl removed the lignin more rapidly than did treatment with Cl in solution although the differences between the lignin contents at corresponding stages were not large.

This problem is being investigated with other lignified tissues to determine the generality and probable cause of the phenomenon.

The methods outlined above were used not only for the preparation in quantity of the fractions for detailed examination but also for preliminary analysis of the powdered shell. The following table gives an average of the analyses of the samples taken from the bulk:

| | % |
|---|---|
| Benzene-soluble | 0·52 |
| Alcohol-soluble | 1·50 |
| Water-soluble | 1·92 |
| 4% NaOH (cold)-soluble | 22·30 |
| Cellulose | 48·04 |
| Lignin | 29·40 |
| | 103·68 |

*Benzene-soluble fraction.* The benzene extract was dark green in colour and showed the absorption bands characteristic of chlorophyll. The residue after the removal of benzene was a brown, stiff gum which failed to solidify even after standing for several months, and was fractionated by dissolving it in boiling 95% alcohol. On cooling, a first, small fraction (A) came down as a hard amorphous mass, the second fraction (B) (about 40 mg.) crystallized after concentration of the solution. On further evaporation a very large part of the chlorophyll was deposited, while the residue (C) was the major part of the original extract and was a brown-coloured, non-drying oil. The following table gives the values obtained:

| | A | B | C |
|---|---|---|---|
| Saponification value | 62·0 | — | 188·0 |
| Iodine value | 15·1 | 4·1 | 73·0 |

The s.v. of "A" suggests that this fraction is a wax and not a fat. The values for fraction "C" are comparable with those (187–197 for the s.v. and 83·2–90·2 for the I.V.) obtained for the crude oil from the embryo of the hazel seed by various workers.

The benzene-soluble material appears to consist in the main of chlorophyll and a non-drying oil with characteristics similar to those of the oil present in the kernel of the nut.

*Alcohol-soluble fraction.* A brown, gummy mass which would not crystallize remained after the removal of the solvent. It was insoluble in water but partially soluble in hot dilute acetic acid, by which, after three treatments, the mass appeared to be completely extracted. The filtrate became opaque on cooling and deposited a precipitate overnight. Both solution and precipitate gave a dark green colour with $FeCl_3$, a brown colour and subsequently a precipitate with potassium dichromate and a green colour with methylene blue, indicating the presence of a tannin of the catechol type. The residue after extraction with hot dilute acetic acid was soluble in dilute NaOH and $NH_4OH$, and reprecipitated on acidification. This fraction was a hard amorphous mass, which melted below 100° and gave no reaction with $FeCl_3$. It appears to be a tannin of the phlobaphene type. The crude alcohol-soluble fraction contained no organic acids, gave negative tests for alkaloids but contained a small quantity of a substance associated with the phlobaphene tannin forming an amorphous 2:4-dinitrophenylhydrazone and an amorphous semicarbazone.

*Water-soluble fraction.* On removal of the solvent a brown residue was obtained. The solution gave negative tests for reducing substances, organic acids, tannins and alkaloids. No recognizable organic material was isolated from it.

*4 % NaOH-soluble fraction.* The three fractions A, A 1 and B, of which the first two made up the major part of the alkali-soluble material, were brown or fawn-coloured amorphous bodies. The pentose and uronic anhydride contents were determined by boiling with 12 % HCl, the methoxyl by Clarke's semi-micro method and the lignin by the method described earlier in this paper. The following table gives the values obtained:

| | A % | A 1 % | B % |
|---|---|---|---|
| Lignin | 26·7 | 58·4 | — |
| Uronic anhydride | 7·6 | 7·2 | — |
| Pentose | 51·4 | 12·0 | 70·2 |
| Methoxyl | 2·6 | 3·7 | — |

The quantity of "B" was too small to permit further examination. 5 g. of hemicelluloses A and A 1 were separately hydrolysed with 16 ml. conc. HCl in 500 ml. water at 100°, the residues filtered off and the filtrates neutralized with $BaCO_3$. Small precipitates separated and were filtered off but yielded no identifiable acids. The filtrate was evaporated to dryness *in vacuo* and the $BaCl_2$ separated from the reducing substances associated with it by the method described previously [Boswell, 1938]. The reducing substances were identified as follows:

| Hemicellulose A | Hemicellulose A 1 |
|---|---|
| Xylose | Xylose |
| Crystals M.P. 142° | Osazone M.P. 155° |
| Cadmium xylobromide crystals | Cadmium xylobromide crystals |
| | Galactose |
| | Mucic acid |

The residues after dilute acid hydrolysis gave the following values:

| | A % | A 1 % |
|---|---|---|
| Fraction of the original | 31·0 | 71·2 |
| Uronic anhydride | 7·8 (2·4) | 3·1 (2·20) |
| Pentose | 12·8 (4·07) | 0·0 |
| Methoxyl | 9·12 (2·81) | 4·7 (3·34) |

The values in the brackets are figures calculated as % of the original material. The methoxyl radical appears to be associated solely with the lignin fractions of the hemicelluloses; this is in accord with the examination of the sugars separated by hydrolysis. Colour tests and absorption spectra indicated the presence of pentoses (HCl and orcinol) and the absence of methyl pentoses (HCl and acetone) and xylose was identified as the only pentose present.

The dilute acid hydrolyses were repeated on the hemicelluloses A and A 1 using 1 % $H_2SO_4$ in place of the HCl in order to test for the presence of organic acids whose Ba salts were soluble in water and insoluble in alcohol, but none was found.

*Cellulose.* The isolated product was a grey powder which in spite of the fact that it gave no red coloration with sodium sulphite solution after exposure to chlorine contained 3·3 % of lignin: pentosan content 23·2 %, uronic anhydride 4·12 % and α-cellulose 48·2 %. The pentosan was isolated by heating the cellulose for 3 hr. with 12 % NaOH at 100°, filtering off the residual cellulose and precipitating by cooling the filtrate to 50° and adding an equal volume of 95 % alcohol. The pentosan accumulated as a granular mass on standing overnight and was separated on a centrifuge. It was hydrolysed by boiling for 1 hr. with 3 % $HNO_3$ [Heuser & Jayme, 1923], the solution neutralized with $BaCO_3$ and evaporated to dryness *in vacuo*. The pentose, after separation from the $Ba(NO_3)_2$

by extracting with absolute alcohol, was identified as xylose by the formation of the characteristic crystals of cadmium xylobromide.

*Lignin.* This fraction was dark brown in colour. Found: C, 60·91; H, 5·36; OMe, 13·01. Calc. for $C_{35}H_{31}O_{13}(CH_3O)_3$: C, 60·64; H, 5·32; OMe, 12·4.

## Discussion

The morphological relationships of the nutshells of *Bertholletia excelsa*, *Arachis hypogea* and *Corylus avellana* have been described previously and it is of interest to note how those of the last two are paralleled by similarities in their chemical constitution and how both differ from the nutshell of *Bertholletia excelsa*. The lignin/cellulose ratio for the hull of the peanut is 0·74 being of the same order as that for the hazel nutshell (0·61) but differing widely from that for the shell of the brazil nut (2·09). The values for the shells of the first two nuts are similar to those for the same ratio for the xylem of the stem of any tree, whether hard or soft wood, e.g. *Fraxinus americana*, 0·54; *Quercus alba*, 0·62; *Pinus ponderosa*, 0·64; and *Sequoia sempervirens*, 0·70. Consideration of the pentosan contents of the hemicelluloses further illustrates this parallelism (*Arachis hypogea*, 12·0%; *Corylus avellana*, about 7·0%; shell of *Bertholletia excelsa*, 0·60%); the corresponding value for the xylem in the stem of any hardwood tree is about 9·0%. Further, the absolute values for the lignin and cellulose without the associated pentosan for *Arachis hypogea* and *Corylus avellana* are in close agreement with those recorded for the xylem in the stem of hardwood trees while those for *Bertholletia excelsa* differ widely from such values.

It would appear therefore that the processes of lignification in the development of secondary xylem in hardwood trees and in the development of the woody pericarps of the fruits of certain of these trees proceed by similar stages resulting in lignified tissues which are alike not only in their lignin/cellulose ratios but also in their lignin and cellulose contents. Further, the hemicellulose fraction of *Corylus avellana*, mainly xylan with a small amount of galactan, is of the same type as that isolated from the secondary xylem of hardwood trees. As both the quantitative and qualitative constitutions of the lignified seed coat of the fruit of *Bertholletia excelsa* differ so widely from those of the tissues just considered it would suggest either that its lignification proceeds by a chain of reactions quite different from that in the lignification of the pericarp and the secondary xylem of hardwood trees, or that a different set of factors determines the constitution of the mature tissue.

These differences do not however extend to the celluloses isolated from the nutshells. The two examined in detail, those from *Corylus avellana* and *Bertholletia excelsa*, are of the same type, both being associated with large quantities of xylan and with relatively large amounts of uronic acid. The cellulose of *Arachis hypogea* is similarly associated with a large amount of pentosan. These celluloses with their large pentosan content and low α-cellulose value are very similar to those isolated from the secondary xylem of the stem of hardwood trees.

The cellulose associated with the lignin in the lignified tissues of hardwood trees would appear therefore to be of the same general form, independent of the morphological identity of the lignified organ, or of differences of constitution in the fractions associated with it, and unaffected by variations in its quantitative relationship to the whole tissue.

In view of our very scanty knowledge of the nature of lignin it is impossible to draw any useful conclusions from the values obtained by elementary analysis of the fractions isolated.

SUMMARY

The pericarp of the fruit of *Corylus avellana* reduced to a fine powder was fractionated with benzene, alcohol, water and 4% NaOH; lignin and cellulose being prepared from the residue. The results obtained from an examination of these fractions show that the morphological identity of the shells of *Arachis hypogea* and *Corylus avellana*, both being the pericarps of their fruits, is paralleled by a similarity in chemical constitution. Both show considerable differences from the shell of *Bertholletia excelsa*, which is the seed coat. It is noted that the lignified pericarps are similar in constitution to the secondary xylem in the stem of hardwood trees and it is suggested that lignification in both tissues proceeds by similar stages but that a different process or set of factors is involved in the development of a lignified seed coat.

My thanks are due to Messrs Gill and Duffus for their kindness in supplying me with the nutshells, to the Trustees of the Dixon Fund of the University of London for the loan of a microchemical balance and to Prof. B. H. Bentley for his interest in the work and for the many ways in which he has facilitated it.

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# CXXXIV. STUDIES ON THE PERMEABILITY OF ERYTHROCYTES

## V. FACTORS IN CATION PERMEABILITY

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Earlier papers in this series [Davson, 1934; 1936; Davson & Danielli, 1936] have been devoted to testing certain published claims regarding the permeability of the erythrocyte to cations [Kerr, 1929; Mond, 1927; Ponder, 1934], some of which have been confirmed and others not. The present work is an attempt to define the conditions under which the erythrocyte may become permeable to $K^+$; so far no systematic investigation of this nature has been made, many observations hitherto described being of a casual or accidental nature, e.g. Ørskov [1935] has shown that the lead dissolved by acid salines from the piston of his syringe was sufficient to cause a rapid loss of $K^+$; Maizels [1935] has shown that human erythrocytes lose $K^+$ in glucose solutions; Kerr [1929] and Davson [1934] have shown that variations in the $Na^+$ and $K^+$ content of the erythrocytes of certain species may occur by varying the nature of the suspension medium appropriately. Of other investigations in the literature those of Yannet *et al.* [1935], Henriques & Ørskov [1936], Eisenman *et al.* [1937] and McCance [1937] are predominantly *in vivo* studies and consequently the range of variation of the nature of the suspension medium is limited to small changes.

The problem has been attacked along three general lines which may be summarized by the questions: (*a*) Is the high concentration gradient of $K^+$ maintained by most erythrocytes against their surroundings due to an active secretory process? (*b*) To what extent may the losses and gains of $K^+$ described in earlier work be due to mechanical damage caused by centrifuging the cells? (*c*) Is haemolysis, however produced, preceded by a preliminary stage of permeability to $K^+$?

Before passing to the experimental part of the paper it should be said that recent work by Davson [1937] indicates that the permeability of the erythrocyte to $K^+$ is to a certain degree a species characteristic, so that for a complete analysis of the conditions under which the erythrocyte becomes permeable to $K^+$ a large number of species should be studied. In view, however, of the wide scope of this paper such was not practicable, so that most of the detailed studies have been limited to the case of the rabbit erythrocyte except where special circumstances demanded the use of other species.

### Experimental

The experimental method was approximately the same in all cases as that of Davson [1934]. A known quantity of blood was measured into a centrifuge tube and the solution with which it was to be washed was added. At the end of the treatment the suspension was centrifuged and the supernatant fluid discarded.

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The cells were haemolysed by the addition of distilled water and the proteins precipitated with trichloroacetic acid. The centrifugate was evaporated and ashed and made up to 10 ml. Duplicate determinations of $K^+$ were made on this solution by the method of Kramer [1920]. The total errors in the procedure rarely aggregated to more than 1 %. It is as well to emphasize that the results are independent of any volume changes which may occur in the erythrocytes, and the method consequently has great advantages over all the others described, and at present being used, by different workers.

## Results

(*a*) *Is there an active secretory process?* The very low oxygen consumption of the non-nucleated erythrocytes of, for instance, the rabbit indicates the absence of an active metabolic process maintaining the concentration gradient of $K^+$ between the cell and its surroundings. Nevertheless Halpern [1936] claims that the migration of inorganic phosphorus across the erythrocyte membrane is determined by the cell glycolysis; further, the observation of Ørskov mentioned earlier might be interpreted as the poisoning of a catalytic system. The effects of a number of catalytic poisons and accelerators on the $K^+$ content of the erythrocytes of the rabbit, goose and dogfish have been tried and the results are shown in Table I. The cells of the goose and dogfish were chosen as they are

Table I. *The effect of narcotic concentrations of certain catalytic poisons and accelerators on the $K^+$ content of erythrocytes expressed as a percentage of the control value. Temperature, unless otherwise stated, 25°. 1 ml. of blood to 10 ml. of suspension medium*

| Species | Narcotic | Concentration | Time hr. | K content |
|---|---|---|---|---|
| Rabbit | NaF | 0·05 *M* | 1 | 100 |
| | NaF | 0·02 *M* | 1 | 99 |
| | NaF | 0·01 and 0·005 *M* | 1 | 100 |
| | NaCN | 0·01, 0·005 and 0·001 *M* | 1 | 100 |
| Goose | NaF | 0·05 *M* | 1 | 95·5 |
| | NaF | 0·02 *M* | 1 | 95 |
| | NaF | 0·01 *M* | 1 | 96 |
| | NaF | 0·005 *M* | 1 | 98 |
| | NaCN | 0·01, 0·005 and 0·001 *M* | 1 | 100 |
| | Ph-urethane | 0·003 *M* | 20 (5°) | 100 |
| | CO | Saturated | 20 (5°) | 100 |
| | NaF | 0·02 *M* | 20 (5°) | 100 |
| Dogfish | NaCN | 0·01 *M* | 10 | 101 |
| | NaF | 0·003 *M* | 10 | 98 |
| | Urethane | 0·001 *M* | 10 | 97 |
| | Methylene blue | 0·02 % | 10 | 101 |
| | Pyocyanine | 0·02 % | 10 | 101 |

nucleated and consequently might be expected to show a more active secretory process. From the table it is seen that of all the poisons studied only fluoride, in the cases of the goose and dogfish, has any significant effect on the $K^+$ content (the effect of urethane is not a narcotic effect but belongs to the phenomena described in the third section). That the effect of fluoride is only remotely, if at all, connected with the inhibition of glycolysis is shown by the extremely small loss of $K^+$ and the fact that the effect increases with increasing concentration of fluoride although all the concentrations studied were adequate to inhibit glycolysis completely. This effect of fluoride, which is very marked at higher temperatures, has been described by Wilbrandt [1937], who informs us that he is

investigating this point and that he is satisfied that it is not due to glycolysis *per se*.

Pyocyanine and methylene blue, in virtue of their effects in increasing metabolism, might be expected to increase the $K^+$ content of the cells suspended in Ringer solutions; actually they had no significant effect on the $K^+$ content of dogfish erythrocytes. Provisionally, then, it may be said that in all probability the high concentration of $K^+$ in the erythrocytes of most species is not maintained by an active metabolic process.

(*b*) *Can centrifuging cause losses of* $K^+$*?* Earlier studies of various workers have shown that the erythrocytes of several species lose $K^+$ when suspended in isotonic salt solutions; in the case of the ox the loss varies between 5 and 15%, appears to be almost instantaneous, and occurs to a lesser extent in serum [Davson, 1934]. In view of the discontinuous nature of this loss it occurred to us that it may be due to the centrifuging of the cell suspension. The erythrocytes of the dogfish, as opposed to those of the ox, have a very high sedimentation rate even in isotonic saline solutions, so that it was found to be quite practicable to wash them with saline or their own serum without centrifuging. In Table II, Exp. 1 it

Table II

| Exp. no. | Species | Treatment | $K^+$ content |
|---|---|---|---|
| 1 | Dogfish | Cells allowed to settle once from serum | 100 |
| | | Cells allowed to settle twice from serum | 100 |
| | | Cells allowed to settle once from Ringer | 100 |
| | | Cells allowed to settle thrice from Ringer | 100 |
| | | Cells centrifuged once from serum | 93 |
| | | Cells centrifuged thrice from serum | 91 |
| | | Cells centrifuged once from Ringer | 96 |
| | | Cells centrifuged thrice from Ringer | 88 |
| 2 | Ox | Cells centrifuged immediately | 100 |
| | | Cells centrifuged after 3·5 hr. | 100 |
| | | Cells centrifuged after 19 hr. | 98 |
| | | Cells centrifuged after 24 hr. | 96 |
| | | Cells centrifuged after 48 hr. | 96 |
| | | Cells centrifuged after 68 hr. | 95 |
| | | Cells allowed to settle from saline | 95·5 |
| | | Cells centrifuged from saline | 88·5 |
| 3 (*a*) | Ox | Cells centrifuged from serum once | 100 |
| | | Cells centrifuged from serum twice | 94 |
| 3 (*b*) | Ox | Cells centrifuged from serum once | 100 |
| | | Cells centrifuged from serum twice | 97 |
| 3 (*c*) | Ox | Cells centrifuged from serum once | 100 |
| | | Cells centrifuged from serum twice | 99 |
| 4 | Horse | Cells allowed to settle from serum | 100 |
| | | Cells centrifuged from serum once | 100 |
| | | Cells centrifuged from serum twice | 100 |
| | | Cells centrifuged from serum thrice | 100 |
| 5 | Ox | Cells centrifuged from serum | 100 |
| | | Cells washed once in isotonic NaCl | 79 |
| | | Cells washed twice in isotonic NaCl | 72 |
| | | Cells washed three, four and five times | 72 |
| 6 | Rabbit | Cells allowed to settle from serum | 100 |
| | | Cells centrifuged from serum | 100 |
| | | Cells washed once in isotonic NaCl | 98·5 |
| | | Cells washed twice in isotonic NaCl | 95·5 |
| | | Cells washed thrice in isotonic NaCl | 95·5 |

is seen that washing dogfish erythrocytes three times, either in their own serum or in Ringer solution, causes no loss of $K^+$ if the cells are allowed to

settle by the force of gravity alone between washings; on the other hand, considerable losses are sustained when the cell suspensions are centrifuged.

Thus in the case of the dogfish, the act of centrifuging the cells alone may cause an appreciable loss of $K^+$. That there should be such a loss is not altogether surprising, for in the centrifuge the lipoid constituents of the plasma membrane will have a centripetal force exerted on them as opposed to a centrifugal force on the erythrocyte as a whole, so that considerable internal stresses will be introduced. This type of leakage by mechanical effects is not of any great theoretical interest, but its practical applications are more noteworthy. Thus in the past, if it was desired to investigate the effect of, for example, time, or a salt solution, on the erythrocyte $K^+$ content, one lot of cells would be centrifuged from serum and ashed immediately for a control, whereas the test lot would be centrifuged from serum with the control lot, resuspended in the test medium and centrifuged a *second time* after the elapse of the desired interval. Obviously, now, the control is not a control, since it has been centrifuged once whereas the test sample has been centrifuged twice. With this in mind the problem of the loss of $K^+$ from the ox cells has been reinvestigated. In Exp. 2 of Table II cells of the ox were allowed to stand in their own serum for varying periods in the ice chest and each sample was centrifuged once only. It is evident that no loss occurs during the first 3–4 hr. and that even after 68 hr. the loss is only 5%. In the same experiment cells were allowed to stand 68 hr. in isotonic NaCl, at the end of which time they had sedimented well enough to remove most of the saline; the $K^+$ content of these cells is compared with that of another lot of cells allowed to stand for an equal time in saline but centrifuged finally. That there is a true escape of $K^+$ during the 68 hr. (4·5%) is evident from the table, but on the other hand the loss obtained when the cells were centrifuged (11·5%) is very much greater. In Exp. 3 (*a*, *b*, *c*) the $K^+$ contents of cells centrifuged once and twice from serum are compared, and here again the same point is brought out, although not so definitely, namely that centrifuging the cells from serum is sufficient to cause a significant loss of $K^+$. In Exp. 4 the absence of a similar effect with horse erythrocytes, which sediment rapidly in their own serum, is shown. The effect of repeated washing of the erythrocytes of the ox and rabbit is shown in Exps. 5 and 6 respectively.

(*c*) *Is haemolysis preceded by a state of cation permeability?* Since this work was begun in 1935, Ørskov has shown that traces of heavy metals may cause a rapid loss of $K^+$ from the erythrocytes of certain species. As heavy metals are strong lysins this fact would appear to substantiate the above hypothesis at least in respect of heavy metal haemolysis; in the present section the effect of a number of different agents, all of which cause haemolysis, on the $K^+$ content of the rabbit erythrocyte is studied. The method adopted is best explained by describing a particular experiment, e.g. that using amyl alcohol. A series of concentrations of this alcohol in isotonic NaCl buffered with phosphate to about *p*H 7·4 were made up in centrifuge tubes and equilibrated in a thermostat at 25°. 1 ml. lots of blood were measured into each tube and after 1 hr. the suspensions were centrifuged and the degree of haemolysis was measured by comparing the colours in the supernatant fluids with those in tubes containing varying concentrations of haemoglobin prepared by diluting a laked specimen of the blood with water. Samples showing a degree of haemolysis above 10% were usually rejected, as the error in the determination of the degree of haemolysis in these cases produced too great an effect on the apparent change in $K^+$ content. Besides the haemolytic concentrations of these substances, just sublytic concentrations were used. If we suppose that 5% haemolysis has occurred in one

sample, then the change in the $K^+$ content of this sample will be 5 % of that of the control if the agent has not caused some or all of the remaining 95 % of the cells to lose $K^+$; if on the other hand the change in the $K^+$ content is greater than 5 % it is concluded that the agent has caused a leakage of $K^+$. In Table III

Table III. *The effect of lysins on the $K^+$ content of rabbit erythrocytes expressed as a percentage of the control values. Corrections have been made for haemolysis so that any value less than 100 indicates an escape of $K^+$. Time 1 hr. Temperature 25°. (The concentrations of digitonin are expressed as fractions of a saturated solution; e.g. "$X/10$" = $\frac{1}{10}$ saturated solution)*

| Lysin | Concentration | % haemolysis | Cell $K^+$ content |
|---|---|---|---|
| Saponin | 1 in 10,000 | 7 | 100 |
| | 0·9 in 10,000 | 4·5 | 100 |
| | 0·8 in 10,000 | 2 | 100 |
| | 0·7, 0·6, 0·5 in 10,000 | None | 100 |
| Digitonin | 4 $X/5$ | 7·5 | 97 |
| | 7 $X/10$ | 5 | 96 |
| | 3 $X/5$ | 3 | 99 |
| | 2 $X/5$ | 1 | 101 |
| | $X/5$ and $X/10$ | None | 100 |
| Na oleate | 2·0 in 10,000 | 10·5 | 93·5 |
| | 1·8 in 10,000 | 8 | 95 |
| | 1·6 in 10,000 | 5 | 97 |
| | 1·4 in 10,000 | 3 | 97 |
| | 1·0 in 10,000 | 2 | 100 |
| | 0·5 in 10,000 | 1 | 100 |
| Na valerate | 0·2, 0·3, 0·4, 0·5 $M$ | None | 100 |
| Na cholate | 4·0 in 1000 | 10 | 96·5 |
| | 1·6 in 1000 | 5 | 100 |
| | 1·0, 0·9, 0·8, 0·7 and 0·6 in 1000 | None | 100 |
| Quinol | 0·075 $M$ | *c.* 5 | 27·7 |
| | 0·050 $M$ | *c.* 5 | 41 |
| | 0·025 and 0·01 $M$ | None | 100 |
| Resorcinol | 0·163 $M$ | None | 16·9 |
| | 0·145 $M$ | None | 33·8 |
| | 0·131 $M$ | None | 62·0 |
| | 0·091 and 0·036 $M$ | None | 100 |
| Catechol | 0·136 $M$ | *c.* 5 | 9·1 |
| | 0·091 $M$ | *c.* 5 | 51·5 |
| *n*-AmOH | 0·18 $M$ | None | 87 |
| | 0·09 $M$ | None | 97 |
| | 0·07 $M$ | None | 100 |
| Guaiacol | 0·04 $M$ | None | 84 |
| | 0·03 $M$ | None | 92 |
| | 0·02 $M$ | None | 96·5 |
| | 0·01 $M$ | None | 99 |
| Guaiacol (dogfish) | 0·01 $M$ ($p$H 5) | None | 85 |
| | 0·01 $M$ ($p$H 6) | None | 86 |
| | 0·01 $M$ ($p$H 7) | None | 83 |
| | 0·01 $M$ ($p$H 8) | None | 83 |
| | 0·01 $M$ ($p$H 9) | None | 85 |
| | 0·01 $M$ ($p$H 10) | 3 | 70 |

the effects of a number of substances which all, with the possible exception of Na valerate, cause haemolysis of the rabbit erythrocyte, are shown. In the final column the $K^+$ content of the erythrocytes has been corrected for the degree of haemolysis, so that any value less than 100 indicates a loss of $K^+$ over and above that due to the actual destruction of the cells. Saponin in both lytic and non-lytic concentrations causes no loss of $K^+$; digitonin, Na oleate and cholate only cause a small loss of $K^+$ when in lytic concentrations, and then only when

Table IV. *The effect of pH and hypotonicity on the $K^+$ content of the erythrocyte expressed as a percentage of the control value. Temperature and time unless otherwise stated 25° and 1 hr. Values have been corrected for haemolysis, if any*

| Species | Treatment | % haemolysis | Cell $K^+$ content |
|---|---|---|---|
| Rabbit | *p*H 6·5–9·0 *c.* | None | 100 |
| | *p*H 6·4 | 1·5 | 100 |
| | *p*H 6·1 | 5 | 99 |
| | *p*H 5·8 | 15 | 97 |
| | *p*H 9·1 (*c.*) | 5 | 99 |
| | *p*H 9·2 (*c.*) | 13 | 94 |
| Dogfish | *p*H 5–9 | None | 100 |
| | *p*H 10 | 3 | 100 |
| Ox | 0·12 *M* KCl 1 hr. (40°) | None | 129 |
| | 0·12 *M* KCl 2 hr. (40°) | 5 | 145 |
| | 0·12 *M* KCl 4 hr. (40°) | 10 | 162 |
| Rabbit | 50° | None | 86 |
| | 55° | 20 | 86 |

the degree of haemolysis is high; of the remaining substances considerable losses may be obtained in both lytic and sublytic concentrations, with the exception of Na valerate.

In Table IV the effects of acidity and alkalinity, temperature and hypotonicity are shown. It is evident that *p*H changes *per se* over the whole of the non-haemolytic range are completely without influence on the $K^+$ content of the rabbit and dogfish erythrocytes. This extends our proof that Mond's [1927] contention that there is a reversal of ionic permeability at alkaline *p*H is groundless [Davson & Danielli, 1936]. In the haemolytic range there seems to be a barely significant loss of $K^+$ when the degree of haemolysis is large (5–15 %), although in the case of a degree of haemolysis of 15 % there is a possible error of about 3 %. Temperature, it will be noted, has a considerable effect even when it is not so high as to cause haemolysis (50°). The effect of hypotonicity has been discussed in detail earlier [Davson, 1937], and the results shown in Table IV are some unpublished ones demonstrating that, besides an escape of $K^+$, penetration of this ion into the cell may also occur from hypotonic KCl. In this experiment ox cells were kept at 40° in 0·12 *M* KCl for 1, 2 and 4 hr. After these periods they were washed in isotonic NaCl so as to remove all intercellular KCl and then the $K^+$ contents were determined. As a result of the penetration of $K^+$ the difference in osmotic pressure between the cells and their suspension medium was increased to such an extent that greater than 10 % haemolysis occurred, whereas a hypotonicity of 0·12 *M* was not sufficient of itself to cause any lysis.

*The effect of silver.* The remainder of this section is devoted to an examination of the effect of the heavy metal silver on the permeability of the rabbit erythrocyte to $K^+$. The silver ion has a strong haemolytic effect on rabbit's blood; concentrations of $1 \times 10^{-3}$ to $1 \times 10^{-8}$ g. per ml. of $AgNO_3$ were tried. After half an hour solutions containing more than $1 \times 10^{-7}$ g. per ml. were found to be haemolytic at 25° so that the studies of this heavy metal have been confined to concentrations of this order. In Table V, Exp. 1, the influence of washing the cells in isotonic $NaNO_3$ on the subsequent effect of silver in a concentration of $1 \times 10^{-7}$ g. per ml. is shown. By adding the unwashed blood directly to the solution containing silver only 7 % of the $K^+$ is lost in 1 hr., whereas after washing twice with isotonic $NaNO_3$ a loss of 66 % of the $K^+$ occurs. In the subsequent experiments the control value of 100 has been ascribed to the $K^+$ content of cells washed twice in isotonic $NaNO_3$ and then suspended in a further

Table V. *The effect of silver on the erythrocytes of the rabbit. Unless otherwise stated 1 ml. of blood to 10 ml. of washing fluid; concentration of $AgNO_3$ $1 \times 10^{-7}$ g./ml.; temp. 25°*

| Exp. no. | Treatment | $K^+$ content | Time min. |
|---|---|---|---|
| 1 | Control | 100 | 60 |
| | $NaNO_3$-$AgNO_3$ added directly to blood | 93 | 60 |
| | ,, ,, to once washed cells | 59 | 60 |
| | ,, ,, to twice washed cells | 35 | 60 |
| 2 | Control | 100 | 60 |
| | Isotonic $NaNO_3$ $2{\cdot}5 \times 10^{-8}$ g. $AgNO_3$/ml. | 96 | 60 |
| | ,, $5{\cdot}0 \times 10^{-8}$ ,, | 94 | 60 |
| | ,, $7{\cdot}5 \times 10^{-8}$ ,, | 83 | 60 |
| | ,, $1{\cdot}0 \times 10^{-7}$ ,, | 51 | 60 |
| | ,, $2{\cdot}5 \times 10^{-7}$ ,, | 46 | 15 |
| | ,, $5{\cdot}0 \times 10^{-7}$ ,, | 35 | 15 |
| | ,, $7{\cdot}5 \times 10^{-7}$ ,, | 27 | 15 |
| 3 | Control | 100 | 45 |
| | Isotonic $NaNO_3$-$AgNO_3$: blood—20 : 1 | 56 | 45 |
| | ,, ,, 10 : 1 | 91 | 45 |
| | ,, ,, 5 : 1 | 100 | 45 |
| | ,, ,, 2 : 1 | 100 | 45 |
| 4 | Control | 100 | 60 |
| | Isotonic $NaNO_3$-$AgNO_3$ unbuffered | 82 | 60 |
| | ,, NaCl-$AgNO_3$ unbuffered | 100 | 60 |
| | ,, $NaHCO_3$-$AgNO_3$ | 91 | 60 |
| | ,, $NaNO_3$-$AgNO_3$-$Na_2HPO_4$ *p*H 6·1 | 80 | 60 |
| | ,, $NaNO_3$-$AgNO_3$-$Na_2HPO_4$ *p*H 7·0 | 93 | 60 |
| | ,, $NaNO_3$-$AgNO_3$-$Na_2HPO_4$ *p*H 7·8 | 93 | 60 |
| | ,, $NaNO_3$-$AgNO_3$-$Na_2HPO_4$ *p*H 8·1 | 79 | 60 |

lot of isotonic $NaNO_3$ for the same time as the test cells are suspended in their silver solutions. In Exp. 2 the effect of varying the concentration of $AgNO_3$ from $2{\cdot}5 \times 10^{-8}$ to $7{\cdot}5 \times 10^{-7}$ g. per ml. is shown, and in Exp. 3 the effect of varying the corpuscle:suspension medium ratio; this latter effect is very striking, since a concentration of silver which may cause 44 % of the $K^+$ to leak out of the cells in 45 min. when they are suspended in 20 ml. of fluid is quite ineffective in causing a leakage when the volume of the suspension medium is reduced to 5 ml. (It should be noted that with $AgNO_3$ in concentrations of greater than $1 \times 10^{-7}$ g. per ml. a certain amount of haemolysis occurred, but in no case was the amount of haemolysis adequate to account for the $K^+$ loss observed.) In Exp. 4 the effect of varying the *p*H of the medium and also of pure isotonic NaCl and $NaHCO_3$ solutions is shown. In the *p*H studies the cells were washed once with isotonic $NaNO_3$, once with the buffer mixture (consisting of four parts of isotonic $NaNO_3$ to one part of isotonic $Na_2HPO_4$ acidified with $HNO_3$) and then suspended in a new lot of the buffer mixture to which $AgNO_3$ had been added. It is clear that there is no definite *p*H in the range studied to the acid or alkaline side of which the effect of silver ceases, and this metal consequently differs from copper in its behaviour in this respect (Davson, unpublished).

## Discussion

The points brought out in section (*a*) need little discussion; it is quite clear that the high concentration of $K^+$ in the cells of the species studied is not maintained by a metabolic process which is normally poisoned by such agents as CN, CO or urethane, or accelerated by methylene blue or pyocyanine; consequently this high concentration gradient must be considered as a static phenomenon.

The effects of centrifuging the cells on their $K^+$ content described in section (*b*) may have an important bearing on the apparent effects of varying the concentrations of $Na^+$ and $K^+$ in the suspension medium on the concentrations of these ions in the cells; as an example Davson [1934] found a 30% loss of $Na^+$ from the ox cells on washing once with isotonic glucose or KCl; Jacobs & Parpart [1933], using an indirect technique which did not involve centrifuging, found only very small losses of total cations so that it may very well be that the loss only occurs when the cells are centrifuged.

By far the most interesting results from the point of view of elucidating the nature of the plasma membrane surrounding the erythrocyte are those given in section (*c*); they also have a bearing on the mechanism of haemolysis. It has been shown that substances such as amyl alcohol, the dihydric phenols, guaiacol etc., cause large losses of $K^+$ in 1 hr. at 25° even in non-haemolytic concentrations; in higher concentrations all these substances are haemolytic, so that it is quite evident that so far as they are concerned a stage of cation permeability precedes the stage of haemolysis. Davson & Ponder [1938] have suggested that the essential element in the process of haemolysis consists in a free permeability of the cell to cations, and these results appear to substantiate the view. Of the remaining lysins studied Na oleate and cholate, digitonin and saponin, the first three show evidence of cation leakage only in haemolytic concentrations (to prevent confusion it should be repeated that in speaking of a cation loss under haemolytic conditions it is meant that there is a loss over and above that resulting from the haemolysis itself), whilst saponin shows no evidence of losses even under these conditions within the range in which it was practicable to study its effects. As this latter group of substances are much stronger haemolytic agents than the former group, there appears to be a contradiction; that this need not necessarily be the case is apparent if it is assumed that a substance like saponin has such a strong effect on the membrane that as soon as it is present in a sufficient concentration to exert its influence, this concentration is sufficient to cause not only a rapid permeability to cations but also haemolysis. In this event no other loss of cations than that due to the haemolysis will be observed. We have formed certain provisional ideas relating to the mechanism of the action of these lysins in causing a permeability of the erythrocyte to $K^+$ but it is not proposed to discuss them in detail until a projected study of the behaviour of thin lipoid films, obtained by extraction of the cells with lipoid solvents, has been carried out; however a bare indication of these ideas would not be out of place. There is a certain amount of evidence that the lipoid constituents of the erythrocyte membrane exist in the surface of the erythrocyte as a solid film; the impermeability of this membrane to cations may therefore depend on the proper orientation of the lipoid molecules, and hence a disturbance of this orientation may lead to a general alteration of the permeability characteristics of the membrane which may include a state of permeability to cations. The haemolytic agents studied above are all substances which might be expected to penetrate the lipoid film and consequently tend to liquefy it, and it may be that the disorientation of the lipoid molecules produced in this way is adequate to cause a permeability to $K^+$. The large effects produced by the substances studied which contain a benzene nucleus, e.g. quinol, may also be correlated with their influence in inhibiting the penetration of glycerol into the erythrocyte; alcohols and other narcotics slow the rate of penetration of glycerol into the erythrocytes of many species; this effect is very much more pronounced with the dihydric phenols (Davson, unpublished); on the other hand substances such as saponin or Na cholate have a very small if any effect. Thus there seems to be

a direct relation between the influences on $K^+$ and glycerol permeability: whether they are causally connected remains to be seen, but it may be remarked here that if a cell is losing $K^+$ it will take longer to haemolyse in a solution of a penetrating non-electrolyte, so that the rate of penetration of the latter will appear by the methods usually adopted to measure it to be slower than it actually is. Before concluding the discussion of these haemolytic agents the effect of guaiacol should be considered more particularly; Osterhout [1930] has founded a theory of the mechanism of accumulation of cations in living cells on the possibility that $K^+$ may enter the cell combined with an organic acid in the undissociated form or as an ionic doublet; the substance used for his experimental models has been this substance guaiacol. At first sight it would appear that the behaviour of guaiacol in causing a loss of $K^+$ from the erythrocyte confirms in some measure Osterhout's theory. That it is wrong, nevertheless, in respect of the erythrocyte, is shown by the complete absence of any effect on the permeability to $K^+$ shown by Na valerate, which should fulfil the requirements demanded by Osterhout's theory more adequately than guaiacol; furthermore the strong effect shown by amyl alcohol, which is not acidic, would make it appear that the loss of $K^+$ is not associated with the power of forming $K^+$ salts, but is due to a more general property of the substances under consideration. The absence of any significant variation of the loss due to guaiacol with *p*H between the limits *p*H 5 to *p*H 9 in the case of the dogfish (Table III) also precludes this view (the increased loss at *p*H 10 is best interpreted as a combination of the separate effects of alkalinity and guaiacol, since a *p*H of 10 is very close to the range of alkaline haemolysis with the dogfish).

It is interesting to note that a change in *p*H of the suspension medium will not cause a loss of $K^+$ until the alkalinity or the acidity is so great as to cause haemolysis, so that the effect of $H^+$ and $OH^-$ is similar to that of the powerful lysins such as digitonin. Temperature haemolysis is preceded by an escape of $K^+$; whether this escape results simply from the increased kinetic energy of the $K^+$ ion or from a change in the membrane as well would be difficult to determine.

In view of the absence of effect of narcotics on the permeability to $K^+$ it would seem that the effect of lead, silver and other heavy metals is not associated with the poisoning of a catalytic system responsible for maintaining a concentration gradient of $K^+$, but may be attributed to a direct influence on the state of the erythrocyte membrane. The influence of heavy metals on negatively charged colloidal systems is, in extremely low concentrations, to favour the aggregation of initially discrete particles. Thus if the normal behaviour and integrity of the erythrocyte membrane are dependent on the maintenance of a certain arrangement of discrete micelles or large molecules, the aggregating influence of a heavy metal such as lead or silver might easily result in the formation of local clumps of molecules or micelles leaving leaky patches in the membrane at other places. On this basis, many degrees of change may be expected, from a mild packing of the molecules or micelles over the whole surface, which might be reflected in an actual decrease in the rate of penetration of certain solutes, to the absolute rupture of the cell membrane, which would be reflected in the phenomenon of haemolysis, according as the concentration and nature of the heavy metal are varied. Davson [1936] showed that should the erythrocyte become permeable to both $Na^+$ and $K^+$, then, as a result of the new Donnan equilibrium conditions, a difference of osmotic pressure should pertain between cells and external medium, which would result in swelling and final haemolysis. Thus the primary mechanism of haemolysis by heavy metals might well be an

initial change in the permeability allowing $Na^+$ and $K^+$ to diffuse through, and resulting in an osmotic pressure difference leading to swelling and bursting of the cells. The consideration of this as a possible factor in haemolytic systems might assist in resolving the complexity of the problem. On the other hand the induced permeability to cations and the haemolysis may represent merely different stages in the same process, so that this mechanism should be looked for only with those low concentrations of heavy metal under the influence of which haemolysis occurs only after some time.

The behaviour of silver in causing a ready penetration of cations leads to some important practical conclusions. Williams & Jacobs [1931] discovered that isotonic solutions of certain "Reagent" brands of NaCl caused the haemolysis of the erythrocytes of certain teleostean fishes, and Ball [1933] showed that the impurity responsible was silver which was introduced during the purification of NaCl. The present work has shown that haemolysis by silver is preceded by loss of $K^+$, so the danger of using such preparations of NaCl even when they produce no visible haemolysis is obvious. Jacobs & Corson [1934] have shown that the rate of haemolysis of certain erythrocytes in glycerol solutions is slowed by the presence of traces of copper (using distilled water from a copper still provides sufficient copper to show the effect) and since then Davson (unpublished) has shown that copper in the same concentrations may cause a leakage of $K^+$; hence in studies on erythrocytes, and presumably on nerve, muscle, marine eggs etc. as well, glass-distilled water and preparations of NaCl known to be free from traces of silver should be used. The practice of defibrinating blood by shaking it with mercury, employed by Yannet *et al.* [1935] and Eisenman *et al.* [1937], should be avoided as any mercury passing into solution would inevitably influence the permeability of the erythrocytes to cations.

## Summary

1. The possibility that the high concentration gradient of $K^+$ maintained by the erythrocytes of many species against their surrounding plasma is due to an active metabolic process has been investigated. Metabolic poisons such as CN, CO, urethane, and accelerators such as methylene blue and pyocyanine had no influence on the $K^+$ content of the non-nucleated erythrocytes of the rabbit and the nucleated cells of the goose and dogfish. Fluoride caused a small loss of $K^+$ but it was concluded that this is not associated directly with the inhibition of glycolysis produced by this substance.

2. The extent to which losses of $K^+$ may be caused by centrifuging the cells from serum or saline was studied. Direct proof of such losses was obtained with the erythrocytes of the dogfish and ox. The possible bearing of these effects on earlier work is discussed.

3. The question as to whether haemolysis, however produced, is preceded by a state of cation permeability was studied. Subhaemolytic concentrations of amyl alcohol, the dihydric phenols and guaiacol caused losses of $K^+$ from the rabbit erythrocyte; digitonin, Na cholate and oleate caused losses only when in haemolytic concentrations (these losses were over and above those due to the haemolysis itself); saponin produced no loss of $K^+$ in all the concentrations studied. Acidity and alkalinity were only effective under haemolytic conditions, whilst raising the temperature caused considerable losses of $K^+$; the penetration of $K^+$ into the erythrocytes of the ox from strongly hypotonic aqueous KCl was demonstrated. The haemolytic action of silver is preceded by an induced permeability to cations.

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# CXXXV. THE ESTIMATION OF CHOLINE AND ACETYLCHOLINE

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THE most specific method of estimating choline consists in acetylation followed by biological assay of the acetylcholine formed [Hunt, 1915]. This method is, of course, as sensitive as that for the estimation of acetylcholine itself, but is very time-consuming. All other methods are chemical ones. Stanek [1904] precipitated choline as the periodide and estimated the iodine by titration with thiosulphate. Roman [1930] advised precipitating the choline as the enneaiodide with a stronger solution of iodine. This method was only applied to serum; I have applied it to other tissue extracts and have found that many substances other than choline are precipitated and the results were as much as ten times too great. In Beattie's [1936] method, which estimates acetylcholine as well as choline, the choline was precipitated as the reineckate and this was dissolved in acetone and estimated colorimetrically. Here again interfering substances increase the result. The method can only estimate 0·3 mg. and it is not possible to estimate acetylcholine in the presence of choline. Mention may also be made of the lengthy method of decomposing the choline to trimethylamine which is then estimated [Linzel & Monasterio, 1931].

The chemical methods for estimating acetylcholine (A.C.) are few and none can be described as a microdetermination. Ammon [1933] estimated A.C. in Warburg's apparatus by means of the carbon dioxide liberated by interaction of enzymically liberated acetic acid with sodium bicarbonate. Abdon & Uvnäs [1937] estimated the acetic acid liberated by choline esterase by adding ferric chloride and estimating the red colour so formed in a step-photometer. The limits of the method were not stated. It should be noted that the above methods depend upon the estimation of the acetic acid formed enzymically from A.C. and, in reality, were developed to study the action of choline esterase.

The method to be described below for the estimation of choline consists first in precipitation as reineckate [Kapfhammer & Bischoff, 1930] and then as the enneaiodide, with elimination of interfering substances at both stages, mainly by differences of solubility of the salts of choline and the interfering compounds. The choline enneaiodide is measured by a slight modification of the usual colorimetric technique.

Addition of iodine to very dilute solutions of choline (or A.C.) caused a deepening of the reddish brown colour of the iodine without formation of a visible precipitate. In the case of choline a visible precipitate did not form until the concentration of choline exceeded 35 $\mu$g. per ml. In the case of A.C. the corresponding value was 20 $\mu$g. It has been found that until a heavy precipitate formed the amount of light passing through the solution was inversely proportional to the concentration of the choline or A.C., provided that the ratio of standard to unknown was not $>2$.

It has been noted by the author and also by Beattie [1936] that choline reineckate has a glistening appearance. This effect is characteristic of choline as it is not given by A.C., lecithin or the interfering substances in tissue extracts. Ammonium reineckate precipitates other substances besides choline from tissue extracts but it has been found that these interfering reineckates are either soluble in $N/10$ NaOH or are insoluble in water at 60°, at which temperature choline reineckate dissolves. Further, the iodides of the interfering substances are insoluble in 30 % alcohol, whereas that of choline is readily soluble.

In the estimation of A.C. it is first necessary to separate the A.C. from choline which is very often present. This presented some difficulties but the separation has been aided by differences in the solubilities of the reineckates and the enneaiodides. A.C. reineckate is soluble in 40 % alcohol in the presence of excess ammonium reineckate, while choline reineckate is only slightly soluble under these conditions. In the case of A.C. it is possible that there is formation of a double salt. On the other hand, choline iodide is more soluble than A.C. iodide in 10 % alcohol. Again in tissue extracts there are other substances which are also precipitated by iodine; most however are precipitated by phosphotungstic acid and the iodides of the remainder are insoluble in 30 % alcohol which, of course, dissolves A.C. iodide. To a certain extent A.C. is precipitated by phosphotungstic acid but only if the concentration exceeds 40 $\mu$g. per ml. and the danger is obviated further by carrying out the precipitation at 30°. Finally, it was found that A.C. required only half the concentration of iodine which was necessary for complete precipitation of choline.

## Experimental

### *Reagents*

(1) *Ammonium reineckate.* A saturated solution is prepared each day by shaking about 5 g. of ammonium reineckate with 100 ml. of water and keeping for 15 min. About 5 ml. of this should be freshly filtered before using.

(2) *Iodine.* 8 g. of iodine and 10 g. of KI are dissolved in 50 ml. of water and kept overnight before use. The solution will keep about a fortnight.

(3) *Approx. N NaOH.*

(4) *Phosphotungstic acid.* A 10 % solution.

(5) *40 % alcohol.*

(6) *Standard choline solution.* The choline is dried at 100° and a solution is prepared so that 1 ml. contains 1 mg.

(7) *Standard acetylcholine.* 0·1 g. of A.C. (Hoffman La Roche) is dissolved in 100 ml. of $N/100$ HCl. This will keep in the cold about a week. It is diluted each day so that it contains 100 $\mu$g. in 1 ml.

The tissue extracts were prepared by dropping the tissue into 10 % trichloroacetic acid (at least 1 ml. per g.), then cutting up finely. The filtered extract was extracted by shaking once with ether, twice with light petroleum and again twice with ether. It was now evaporated under reduced pressure at 40° till 1 ml. was equivalent to 1–2 g. of tissue.

### *The estimation of choline*

A standard reineckate suspension was prepared by adding 0·3 ml. of ammonium reineckate to 1 ml. of a solution of choline containing approximately 20 $\mu$g. It was then centrifuged and the supernatant fluid poured off and the precipitate was suspended in 1 ml. of water. This should keep several hours. To

1–5 ml. of choline solution or tissue extract, which should be neutral or slightly acid, was added half the volume of ammonium reineckate and the solution was then shaken; in the case of tissue extracts 0·1–0·5 ml. of *N* NaOH was added (for 1–5 ml.). It was then centrifuged for 2 min. at 3000 r.p.m., the liquid was poured off and the tube was drained as much as possible, care being taken that none of the precipitate was lost. Water was now added, 1 ml. at a time, with shaking, till a suspension of the same density as the standard reineckate previously prepared was obtained. The volume added was noted and the tube was placed in a water bath at 60°. After 2 min. it was centrifuged for 2 min. and the fluid poured into another tube; 0·3 ml. of the iodine reagent was now added and the tube compared with a standard in the colorimeter. 0·3 ml. of alcohol was added to the unknown and was again compared. The correct value was obtained by subtraction. The standard was prepared by taking 1 ml. of a solution containing 25 $\mu$g. of choline to which was added 0·5 ml. of ammonium reineckate. It was then shaken and centrifuged, the fluid was poured off and 1 ml. of water was added to the precipitate which was now heated to 60°, cooled and 0·3 ml. of iodine added as before. There are several points about this estimation of choline which may need explanation. The preparation of the standard suspension of 20 $\mu$g. of choline reineckate was a device to obtain the choline of the unknown in a dilution at which the estimation by the method suggested was most accurate. The alcohol was added to the iodide to dissolve the choline iodide, but it did not dissolve iodides other than those of choline. It has been found that the colour due to a solution of about 20 $\mu$g. of choline iodide in 1 ml. was reduced to that of the colour of the iodine alone by the addition of 0·3 ml. of alcohol.

Lastly, the method did not distinguish between choline and acetylcholine and so, strictly speaking, estimated total choline. Of course the acetylcholine, if present, could be estimated by the method to be described and the choline itself could be obtained by difference.

### *The estimation of acetylcholine*

A. *Pure solutions or extracts which contain a minimum of interfering substances such as serum, which should contain not more than 400 $\mu$g. of choline.*

It is first necessary to determine the approximate choline content by the above method.

In 1·5 ml. the total choline content was brought to 400 $\mu$g. by addition of the necessary amount in the smallest volume possible (about 0·5 ml.). At the same time a control blank was carried out with 400 $\mu$g. of choline alone. Half the volume of ammonium reineckate was added to both tubes. They were then shaken and centrifuged for 2 min. The supernatant liquid was poured off and the tubes were drained as much as possible, then 1·4 ml. of 40 % alcohol and 0·2 ml. ammonium reineckate were added to the tubes which were shaken to disturb the precipitate and placed in a water bath at 57° for 3 min. They were centrifuged again and the liquid poured into other tubes which were now placed in a vigorously boiling water bath for 4 min. The tubes will boil easily if a "boiling" tube is added (a capillary sealed about 2·3 cm. from one end; these tubes should always be removed from the solution while it is boiling and then they will last indefinitely). The tubes were cooled and 0·1 ml. of the iodine reagent was added (a fairly heavy precipitate should form), followed by 0·05 ml. of alcohol and the tubes were inverted once. If the precipitate in the control tube did not almost completely dissolve a further 0·03 ml. of alcohol was added to all tubes,

and if this were not sufficient another 0·03 ml. Care must be exercised at this point. On addition of the alcohol to the control solution it should become almost, but not definitely, clear; it usually takes about 30 sec. for the alcohol to dissolve the choline iodide. If too much alcohol is added A.C. may be lost. The tubes were centrifuged and the liquid poured off, 0·04 ml. of alcohol was added to the tubes and the precipitates were dissolved by placing the tubes momentarily in hot water; 1 ml. of water and 0·15 ml. of iodine were added and the tubes were compared with a standard in the colorimeter. The reading for the blank was then subtracted.

The standard was prepared by adding 0·15 ml. of iodine and 0·04 ml. of alcohol to 1 ml. of a solution containing 5–15 $\mu$g. of A.C. as required.

The solution to be tested should contain between 4 and 20 $\mu$g. of A.C. although amounts down to 1 $\mu$g. can be estimated. If the solution should contain more than 20 $\mu$g. the test should be repeated with a more dilute solution.

The standard deviation is $\pm 0{\cdot}68$ $\mu$g.

It has been found empirically that small amounts of A.C. are only precipitated completely under the conditions employed if a definite amount of choline is present; 400 $\mu$g. have been chosen as this amount. In addition the temperature at which the mixed reineckates of choline and A.C. dissolve in 40% alcohol varies with the amount of choline present and the age of the ammonium reineckate. If the solution contains more than 400 $\mu$g. of choline the temperature at which the A.C. reineckate is dissolved should be raised 3° for each additional 50 $\mu$g. of choline. If on the addition of 0·1 ml. of iodine to the control tube no precipitate forms it is best to repeat from the beginning, raising the temperature to 60°. If, on the other hand, the control solution does not almost clear on the addition of 0·11 ml. of alcohol the temperature should be reduced to 54°. However, in most cases 57° will be found to be most suitable. The need for varying the temperature has been found because the ammonium reineckate is not stable and various samples differ from one another. Apparently this affects the ease of solubility of the A.C. reineckate and so some samples require a higher temperature than others.

B. *Tissue extracts containing interfering substances, such as liver, stomach and intestinal extracts.*

The approximate choline content of the extract should be determined and if it is less than 30 $\mu$g. choline should be added to this amount. This prevents an excess of phosphotungstic acid which may precipitate some of the A.C. If there are more than 90 $\mu$g. of choline present the amount of phosphotungstic acid to be added should be increased by half. To 1 ml. of extract which had been in a water bath at 30° for 2 min., 0·1 ml. of phosphotungstic acid was added immediately and the tube was inverted but not shaken and then centrifuged for 2 min. The liquid was poured carefully into another tube and 0·15 ml. of iodine and 0·08 ml. of alcohol were added. It was then centrifuged for 2 min., the liquid poured off and 0·04 ml. of alcohol was added, the tube being warmed as before; then 1 ml. of water and 0·15 ml. of iodine were added. It was read in the colorimeter and then 0·25 ml. of alcohol was added and it was read again. The value of A.C. was obtained by subtraction.

The amount of light passing through the solution is inversely proportional to the concentration within the limits of 4–25 $\mu$g. of A.C. provided that the ratio of standard to unknown does not exceed 2. Below 4 $\mu$g. the standard should more nearly approach the unknown. As the standard takes only a few seconds to prepare it is not a difficult matter to make several to compare with the one unknown.

If a scum should form on the surface of any of the tubes it can be carried down by adding two drops of a suspension of aluminium hydroxide. This was prepared by precipitating 25 g. of potassium alum in water with 5·5 g. NaOH. It was filtered, washed and made into a suspension with 80 ml. of water.

*Results with tissue extracts*

The results in Table I give the choline and A.C. contents of various tissues. In addition are given the results obtained for A.C. by estimation on the frog's rectus abdominis or the leech muscle.

Table I

| Tissue | Animal | Choline μg./g. | A.C. μg./g. Chemical method | A.C. μg./g. Biological method |
|---|---|---|---|---|
| Submaxillary gland | Dog | 6·0 | 4·2 | — |
| Small intestine | Dog | 48 | 3·0 | 2·8 |
| | | 62 | 3·2 | 3·1 |
| | Rabbit | 20 | 2·0 | 2·1 |
| | | 32 | 2·5 | — |
| | Cat | 32 | 0·5 | 0·5 |
| | | 21 | 0·5 | — |
| Stomach | Dog | 10 | 1·0 | 1·0 |
| | | 12 | 1·5 | — |
| | Rabbit | 12 | 1·8 | 1·6 |
| | | 14 | 1·2 | 1·0 |
| | Cat | 25 | 1·2 | 0·9 |
| | | 16 | 0·05 | — |
| Liver | Dog | 70 | — | 0·1 |
| | | 64 | — | — |
| | Rabbit | 30 | — | — |
| | | 32 | — | — |
| | Cat | 95 | 0·5 | 0·2 |
| | | 74 | — | 0·1 |
| Blood | Rabbit | 1·0 | — | — |

In addition some of the tissue extracts were acetylated and tested on the leech muscle. The results so obtained agreed with those for choline obtained by the chemical method.

*Recovery of added* A.C.

A.C. was added in varying amounts to the concentrated extract and the usual procedure carried out. In all cases, with the exception of some intestinal extracts, the recovery was 90–100 %. Often it was found impossible to obtain intestinal extracts which were perfectly clear; whether the particles were fat or breakdown products of protein is difficult to say. If the latter were true one might have expected adsorption to have taken place, for the recovery of A.C. varied from 70 to 90 %.

SUMMARY

Sensitive and specific methods for the estimation of choline and acetylcholine are described.

The sensitivity of the method for acetylcholine is 1 in $10^6$, of that for choline 5 in $10^6$.

The methods have been applied to tissue extracts and give results which agree with those obtained by biological methods.

I wish to thank Prof. J. H. Gaddum and Dr H. R. Ing for continued help and interest throughout the course of the work.

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# CXXXVI. THE DETERMINATION OF VITAMIN A WITH THE PHOTOELECTRIC COLORIMETER

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THIS communication describes the application of the Evelyn photoelectric colorimeter [Evelyn, 1936] to the quantitative estimation of vitamin A by the antimony trichloride reaction of Carr & Price [1926]. It was thought that the Carr-Price test with its attendant difficulties due chiefly to the instability of the colour to be measured (for data on this point, see below) might be performed more accurately with the photoelectric colorimeter on account of the rapidity with which readings can be taken on this instrument. The results described below show that the Carr-Price test can be performed with great precision by this method.

Apart from the biological assay there are two methods, one physical and the other chemical, which have been widely used for the estimation of vitamin A. The physical method is based on the discovery of Takahashi *et al.* [1925] that vitamin A concentrates show selective absorption in the ultraviolet spectral region with a maximum at 328 m$\mu$. This method was introduced by Drummond & Morton [1929], and its status as a fairly satisfactory method is based on critical examination by Coward *et al.* [1931], Morgan *et al.* [1935], the Medical Research Council [1935] and Hume [1937].

In the chemical method of Carr & Price [1926], the vitamin A is made to react with antimony trichloride in chloroform solution, the amount of the resultant blue pigment being determined by examination of the solution in a tintometer or visual spectrophotometer. The use of the tintometer is attended by considerable error owing to the unavoidable difficulties in colour matching by eye, and also to the peculiar physical basis of tintometry which is responsible for the non-linear relation between the tintometer reading and the amount of vitamin A [Notevarp & Weedon, 1936]. Spectrophotometric measurement shows, however, that the extinction coefficient of the blue solution at 620 m$\mu$ is a linear function of the concentration of vitamin A in any given material [Notevarp & Weedon, 1936]. That the chromogen which reacts with antimony trichloride to produce the blue colour is vitamin A itself is indicated by the observations of Holmes & Corbet [1937] who found that a crystalline preparation of very high biological potency gave the characteristic reaction. In spite of the considerable inaccuracies inherent in methods available for measuring the intensity of the blue colour up to the present, it has been shown that the results of this chemical method are almost as closely correlated with the biological assay as are the results of the physical method of estimation [Morgan *et al.* 1935; cf. Table VI, p. 1654]. Thus the chemical and physical methods have equally sound foundations as shown by earlier observations.

In the past the investigator has therefore had the choice of using the inaccurate tintometric chemical method requiring little capital outlay, or the more accurate spectrophotometric chemical or physical methods requiring expensive apparatus (and for the physical method consuming much time in the exposure, development and reading of the photographic plates). Recently a special photoelectric photometer utilizing the physical method has been described [McFarlan *et al.* 1937]. This instrument permits rapid measurement of the extinction coefficient at 328m$\mu$, but has the disadvantage that it cannot be applied to uses other than the estimation of vitamin A.

There is at present no evidence that measurement of absorption at 328m$\mu$ is any more closely related to biological activity than is the depth of the blue colour produced in the Carr-Price reaction, but it is generally agreed that the former quantity is more susceptible of accurate measurement than the latter as long as one is forced to use tintometers or visual spectrophotometers for examination of the blue colour. The advantages of the photoelectric colorimeter[1] [Evelyn, 1936] for the measurement of the very unstable blue colour of the Carr-Price reaction may be readily appreciated by the following brief description of the apparatus, which is essentially a single photocell direct-reading photoelectric photometer equipped with light filters. A beam of approximately monochromatic (filtered) light is directed upon a photoelectric cell connected to a galvanometer which records the intensity of the incident light directly on a scale of 100 divisions. An absorption cell containing the solution under test may be interposed between the lamp and the photocell, and the ratio of the final and initial galvanometer readings measures the light transmission of the coloured compound. Important features of the design of the apparatus, in addition to simplicity, rapidity and objectivity of operation, are extreme constancy of the light source, the use of filters of high selectivity and permanence of spectral characteristics, and strictly linear relation between galvanometer deflexion and light intensity. For the vitamin A determination it is also important that the colorimeter employs a test tube type of absorption cell the open end of which projects above the top of the apparatus, thus allowing reagents to be added to the test solution while the tube is actually in place in the path of the beam of light. This feature together with the direct reading nature of the device enables the operator to record at short intervals the development and fading of the colour for any period he desires beginning within a second or two after addition of the reagent, and makes it possible to measure the intensity of the blue colour at its true maximum which, as we shall show below, occurs immediately after addition of the reagent.

Another important component of the photoelectric colorimeter is the filter which is used to isolate a narrow spectral region in the vicinity of the region of maximum absorption of the solution under test. Since a complete set of highly selective filters has been developed as an integral part of the apparatus, one has no difficulty in selecting a filter (No. 620) to fulfil this requirement for the vitamin A-antimony trichloride complex. Fig. 1 shows spectrophotometric transmission curves of the blue compound and of the filter used in its determination.

[1] The application of the Evelyn photoelectric colorimeter to the measurement of vitamin A was first suggested by Dr Charles May of the Children's Hospital, Boston, Massachusetts. Dr May has perfected a micro-method for the determination of vitamin A in serum, which is described in a paper now in the press.

The colorimeter including all the necessary filters can be obtained from the Rubicon Company, 29 North Sixth Street, Philadelphia, Pa., U.S.A.

It should be emphasized at this point that the physical basis of photoelectric measurements made with spectral filters rests on an accurate mathematical foundation no less secure than that of conventional spectrophotometric determinations. The relation between the true spectrophotometric transmittance $T_\lambda$

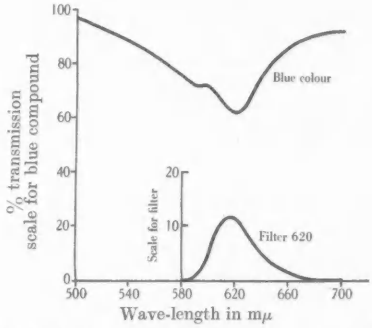


Fig. 1. Spectral characteristics of the vitamin A antimony trichloride blue colour and of the filter employed in its measurement.

at any wave-length $\lambda$, and the average transmittance $T_\lambda'$ which is measured with the corresponding filter is given by the equation

$$T_\lambda' = \frac{\Sigma_{\lambda_1}^{\lambda_2} T_\lambda F_\lambda . \delta\lambda}{\Sigma_{\lambda_1}^{\lambda_2} F_\lambda . \delta\lambda}, \qquad \text{.....(1)}$$

where $\lambda_1$ and $\lambda_2$ are the limits of transmission of the filter, and $F_\lambda$ is the transmittance of the filter at wave-length $\lambda$. These integrals can be evaluated rapidly and accurately from the known spectrophotometric curves of the coloured compound and filter by graphic representation and the use of the planimeter.

Finally it may be added that the filters are always made sufficiently selective to fulfil the requirement that the concentration of the coloured compound shall be proportional (within the limits of error of the determination itself) to the negative logarithm of $T'$ as well as to that of $T$, thus allowing the use of standard spectrophotometric formulae in calculation of results obtained with the photoelectric colorimeter. To prevent confusion of terminology the following symbols are used throughout.

$$E = -\log_{10} T \text{ (spectrophotometer)},$$
$$L = -\log_{10} T' \text{ (photoelectric colorimeter)}$$
$$= -\log \frac{G}{100} = 2 - \log G,$$

where $G$ is the galvanometer reading obtained on inserting the sample solution, the initial adjustment of the light intensity having been made to give a reading of 100 through a tube containing pure solvent only.

*Procedure.* The method is applicable to any fatty material containing vitamin A. If the vitamin A content of the material is small (as in cod liver oils) then the fat must be saponified and the unsaponifiable residue used for the test. This eliminates interference of unsaturated fatty acid radicals with the Carr-Price reaction [Emmerie, 1933]. It is suggested that saponification should be carried out whenever the vitamin A content of the material is believed to be less than 10,000 I.U. of vitamin A per g.

A weighed amount of the fat or oil (about 250 mg. for a cod liver oil) is boiled in a small flask for 2 min. with 2 ml. of 60% aqueous KOH and 10 ml. of 95% alcohol. The resulting solution is poured into 10 ml. of distilled water in a separating funnel, and the flask washed with two lots of 15 ml. of water and two lots of 25 ml. of ether, all being poured into the separating funnel. The funnel is shaken vigorously and the aqueous layer drawn off and discarded. The ethereal solution is washed vigorously with 10 ml. of water and then twice gently with 25 ml. of water each time and finally is dried by filtration through anhydrous sodium sulphate on a sintered glass diaphragm, and the sulphate washed with 20 ml. of ether. The ethereal solution is evaporated to dryness by connecting the containing flask directly to a water pump without using a condenser, the flask being immersed in warm water until the ether has evaporated, and then dipped for a moment in boiling water to drive off traces of water from the unsaponified residue.

The residue is dissolved in 25 ml. of chloroform, a suitable aliquot (say 0·25 ml. for first trial) is pipetted into one of the special photocolorimeter absorption test tubes, and the volume is made up to 1 ml. with chloroform. For a richer oil when saponification is unnecessary a few mg. should be weighed out and made up to a known volume with chloroform; a suitable aliquot of this solution is then transferred to the colorimeter tube. The appropriate filter (No. 620) is placed in the colorimeter, the apparatus is turned on, and the light intensity is adjusted so that a galvanometer deflexion of 100 divisions is obtained through a tube containing 1 ml. of chloroform and 9 ml. of reagent (or 10 ml. of reagent if more convenient, as it gives the same result). This blank tube is then replaced by the sample tube and 9 ml. of the antimony trichloride reagent (25 g. per 100 ml. of solution) are run rapidly down the side of the tube from an automatic pipette of the B.D.H. pattern or other suitable type[1] of automatic pipette or burette.

As the reagent and test solution mix the galvanometer spot will move across the scale to a new position of temporary equilibrium which is recorded by the operator as $G_{620}$. If the reading is less than 20 or more than 70, another tube should be set up with a correspondingly smaller or larger volume of the chloroform solution.

The "$L$ value" of the oil may then be calculated from the equation

$$L \text{ value} = \frac{52{\cdot}5}{M_{620}} \times L_{620}, \qquad \text{.....(2)}$$

where $L_{620} = (2 - \log G_{620})$, and $M_{620}$ mg. is the weight of original material yielding the aliquot of unsaponifiable residue placed in the colorimeter tube.

*Derivation of the new unit.* The unit adopted for expression of results by the new method has been chosen to be as closely as possible analogous to the familiar $E$ (1%, 1 cm. 620m$\mu$) and is written $L$ (1%, 1 cm. 620m$\mu$). It is usually referred to as the "$L$ value". If $M$ mg. is the amount of original material corresponding to the volume of the chloroform solution used in the test, then the concentration of original material in the final blue solution is $M/10$ mg. per ml. or $M/100$ g. per 100 ml. Bearing in mind that the effective thickness of the layer

[1] The essential characteristic of whatever device is used for the delivery of the reagent solution is that its delivery time shall be no longer than 2 sec. The reading obtained is the same whether (*a*) the reagent is run into the tube already in position in the colorimeter, or (*b*) the reagent is run into a tube held in the hand, the tube rapidly shaken and inserted into the colorimeter. This shows that complete mixing occurs rapidly when the reagent is run into the tube already in the colorimeter.

of solution through which the light passes in the tubular photocolorimeter cell is 1·9 cm. it is clear that the quantity which we have referred to above as $L_{620}$ is more exactly $L$ ($M$/100%, 1·9 cm., 620mμ) and therefore

$$L \text{ value} = L\,(1\%,\ 1 \text{ cm.},\ 620\text{m}\mu) = \frac{100}{M_{620}} \times \frac{1}{1{\cdot}9} \times L_{620}$$
$$= \frac{52{\cdot}5}{M_{620}} \times L_{620}. \qquad \text{.....(2)}$$

By calculation from equation (1) it has been found that the ratio of $E$ (1%, 1 cm., 620mμ) to $L$ (1%, 1 cm., 620mμ) is 1·30 ± 0·03.

*Correction for the presence of carotene.* Since in the presence of antimony trichloride carotene develops a colour similar to that given by vitamin A, a correction for carotene present should be applied to the $L$ value when the chloroform solution is seen to be yellow. To do this, 10 ml. of the solution are pipetted into another colorimeter tube, filter 620 is replaced by filter 440, and after adjusting the galvanometer reading to 100 with a blank tube containing 10 ml. of chloroform the galvanometer reading with the tube of solution in place is recorded as $G_{440}$. If the reading is less than 20, a suitable dilution of the solution is made to obtain a reading between 20 and 70.

The "corrected $L$ value" of the oil may then be calculated from the equation

$$L \text{ value (corrected)} = 52{\cdot}5\left(\frac{L_{620}}{M_{620}} - 0{\cdot}14.\frac{L_{440}}{M_{440}}\right), \qquad \text{.....(3)}$$

where $L_{440} = (2 - \log G_{440})$, and $M_{440}$ mg. is the weight of original material yielding the aliquot of unsaponifiable residue contained in the tube used for recording $G_{440}$.

The observations which establish the constant 0·14 used in this equation are described below.

## *Experimental*

*Application of laws of Lambert and Beer to the blue solution, and precision of the method.* It is clear that the definition of the new unit involves the assumption that $L$ (the negative logarithm of the transmittance) is a linear function of $M$

Table I. *Data on the precision of estimation of vitamin A with the photoelectric colorimeter. Readings of L for different volumes of the same solution containing vitamin A.*

| Volume of solution (ml.) | $L$ | $L \div$ volume |
|---|---|---|
| 1·0 | 0·601 | 0·601 |
| 0·95 | 0·572 | 0·602 |
| 0·90 | 0·542 | 0·602 |
| 0·85 | 0·520 | 0·611 |
| 0·80 | 0·485 | 0·606 |
| 0·75 | 0·457 | 0·609 |
| 0·70 | 0·424 | 0·606 |
| 0·65 | 0·3955 | 0·608 |
| 0·60 | 0·365 | 0·608 |
| 0·55 | 0·3355 | 0·610 |
| 0·50 | 0·302 | 0·604 |
| 0·45 | 0·274 | 0·609 |
| 0·40 | 0·246 | 0·615 |
| 0·35 | 0·214 | 0·612 |
| 0·30 | 0·1845 | 0·615 |
| 0·25 | 0·152 | 0·608 |
| 0·20 | 0·119 | 0·595 |

Mean value of $L \div$ volume = 0·607.
Standard deviation of $L \div$ volume = ±0·00515 = ±0·85%.

(the concentration of vitamin A). In other words it is assumed that Beer's law governs the relation of depth of blue colour produced to amount of vitamin A present. Notevarp & Weedon [1936] have shown this assumption to be valid for extinction coefficients measured on the spectrophotometer, and the results given in Table I prove that the photocolorimeter readings also satisfy this relation to a high precision. The procedure was as follows.

A solution was made of an unsaponifiable residue from cod liver oil and the solution was measured into specially calibrated colorimeter tubes from a microburette graduated in intervals of 0·01 ml. Care was taken to wipe the burette tip before each delivery in order to remove unsaponifiable residue deposited there by seepage of the solution past the closed tap and evaporation at the tip. The solution in each tube was made up to 1 ml. by addition of pure chloroform from a similar microburette.

The solvent used was dry alcohol-free chloroform which had been distilled 5 days previously, and the reagent was a 20 % solution of antimony trichloride (throughout this paper the concentration of antimony trichloride solutions are given as percentages which signify g. solute in each 100 ml. of solution) made up 2 days previously in some of the same batch of chloroform. All readings were taken at 20°. Each reading was taken once only; no duplicates were read. From the figure given for the standard deviation it appears that when working with this degree of care only 1 observation in 22 will err by more than $\pm 1{\cdot}7$ % from the true value of $L$.

These figures show how small is the error of the method, which includes both the error of transferring a measured aliquot of test solution to the colorimeter tube and the personal and instrumental errors in the galvanometer reading. Unless the closest attention is paid to the measurement of the volume of test solution, this degree of accuracy cannot be obtained, as a much larger error will be made in this operation. In any reading this error is likely to control the size of the error of the complete process of taking the reading.

*Concentration and purity of the reagent.* Fig. 2 shows the effect of varying the concentration of the solution of antimony trichloride. Carr & Price [1926] originally suggested using a 30 % solution in chloroform. In our experience a

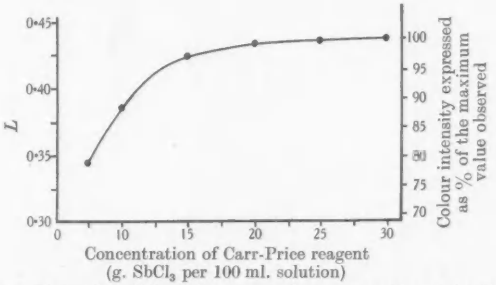


Fig. 2. Dependence of intensity of blue colour upon the concentration of the Carr-Price reagent. Each reading was made by treating 1 ml. of a standard solution of vitamin A with 9 ml. of Carr-Price reagent of the concentration shown by the individual point.

solution of this concentration can be prepared from reagent quality antimony trichloride and reagent quality chloroform by warming, and the solution will generally remain stable down to 20° at least; but if the driest commercial specimens of antimony chloride are chosen and dissolved in pure dry alcohol-free

chloroform at this concentration, then much of the salt will crystallize out at 20°. After the solution and solid have been standing for some weeks, however tightly stoppered (in dark or light), the salt can be redissolved by warming and will not again crystallize out on keeping at 20°. The data charted in Fig. 2 were therefore obtained with a stale reagent.

Fig. 2 shows that there is no need to increase the reagent concentration above 20%, and that the concentration need not be very accurately controlled. This is a great advantage in the use of such a hygroscopic reagent.

Carr & Price [1926] stated that traces of water and alcohol do not affect it deleteriously. This statement was denied by Notevarp & Weedon [1936] who found that water, alcohol or degradation products produced by the action of light on the chloroform all affected the depth of blue colour produced. In our experience the former authors are correct, as shown by the following observations. A fresh 20% solution (solution A) of antimony trichloride in pure dry alcohol-free chloroform was prepared. From this solution were prepared a solution B consisting of 99 ml. solution A + 1 ml. alcohol; solution C, 95 ml. of solution A + 5 ml. alcohol; and solution D, 99 ml. of solution A + 1 ml. water. Solution D became cloudy, but on standing it separated into two clear layers, the lower very small in volume. Only the upper layer was used for this test. Solution E was a 20% reagent solution which had been standing in a corked flask in a bright room for 6 months and had separated into large upper and small lower immiscible layers. The upper layer was tested. When each of these solutions was used as reagent with 1 ml. of the same solution of vitamin A, triplicate readings were obtained which were identical within the limits of error of the method. Thus for the vitamin concentrations permissible in this method stale reagents or reagents containing traces of water, alcohol or degradation products do not affect the initial depth of the colour produced. This again is very convenient since the reagent solution is hygroscopic and unstable.

*Fading of the blue colour.* The greatest difficulty in spectrophotometric measurement of the blue colour is due to the rapidity with which the colour

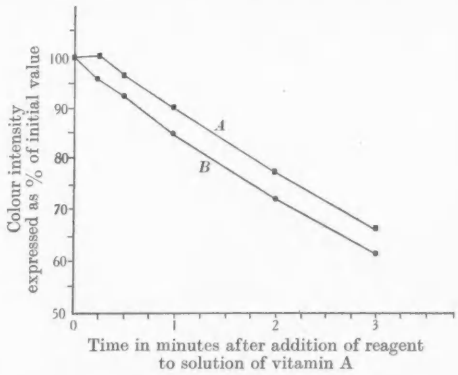


Fig. 3. Observations on the rate of fading of the blue colour. Curve *A* was obtained by using a freshly prepared reagent solution; curve *B* with a stale reagent solution. The initial readings with each reagent were identical.

fades. This difficulty is completely overcome by the photoelectric colorimeter, as the reading on any test solution can be taken within 2–3 sec. of adding the

reagent; this allows time for the mixing of the test and reagent solutions and the swing of the galvanometer spot.

Carr & Price [1926] claimed that the blue colour is "stable" for 3 min. after its appearance, but our experience shows on the contrary that the colour begins to fade at once if a stale reagent solution is used, and after 10–15 sec. if a fresh solution is used. When a stale reagent solution is added to a test solution in a tube already in position, the galvanometer spot moves rapidly to an initial reading and then immediately begins to drift slowly to the right, indicating a decreasing intensity of colour. But with fresh pure reagent solution the spot moves equally rapidly to the same initial position and then remains motionless for 10–15 sec. before it begins to drift away. Thus there is a real advantage in using the fresh pure reagent solution as the reading can be noted in more leisurely fashion. Moreover, it allows a test to be made of the thoroughness with which mixing occurs in the tube when the reagent is added, as there is time to add the reagent and shake the tube well before placing it in position. This test shows that complete mixing occurs very rapidly when the reagent is added to the tube already in position.

The rate of fading of blue colour produced from one and the same test solution with fresh and with stale reagent solutions is shown in Fig. 3. The rate of fading appears to vary somewhat with the particular test material, but in our experience the difference between the fresh and stale reagents is always as shown in Fig. 3. Similar fading occurs whether the test solution contains an oil or an unsaponifiable residue.

*Effect of temperature.* We have confirmed earlier observations that temperature exerts an effect on the depth of colour produced, but no accurate measurements have been possible. Readings were taken in a warm room, and the instrument and solutions transferred to a refrigerator where further readings were taken after some hours. Measurements were first made at 21° and 11° respectively, and the $L$ value was found to be 4·8 % greater at the lower temperature. Later another test was made at 29° and 5° and $L$ was observed to be 10·6 % greater at the lower temperature. From these figures it seems that the observed value of $L$ is increased by about 4–5 % for a temperature decrease of 10° within the range 0–30°: this is much less than was claimed by Wokes & Willimott [1927], but a careful reading of their paper shows that there was some confusion between depth of colour produced and the rate at which it developed.

*The chromogenic power of carotene relative to its yellow colour.* The following observations were made in order to calculate the numerical factor employed within the bracket of equation (3) above. 23·3 mg. of commercial $\beta$-carotene were weighed from a freshly opened ampoule and dissolved in 10 ml. of chloroform. Further dilutions were made and $L_{440}$ for each of the resultant solutions was measured. It was found that the solutions obeyed Beer's law with precision and the $L_{440}$ value of the $\beta$-carotene was 1440. A series of lesser dilutions was also prepared and to 1 ml. of each solution 9 ml. of the 20 % antimony trichloride were added and $L_{620}$ measured for each. It was found that the blue solution obeyed Beer's law, and the $L_{620}$ value of the carotene was 220.

The ratio of $\frac{L_{620}}{L_{440}}$ was $\frac{220}{1440}$ or 0·153.

Similarly with a commercial specimen of carotene stated to contain 25 % of $\alpha$-carotene the values observed were $L_{440}=1641$, $L_{620}=220$ and $\frac{L_{620}}{L_{440}}=0{\cdot}130$. The absolute values of $L$ recorded here are undoubtedly too low on account of the difficulty of obtaining pure specimens of carotene; we hope to be able to prepare pure specimens and redetermine the figures in the future. The ratios are likely

to be more accurate, and for the present the mean value of the two determinations is suggested for use in equation (3).

*Relation of L value to the other units.* Since it was felt that the present method has materially increased the precision and accuracy of the measurement of the blue vitamin A-antimony trichloride complex, it appeared desirable to determine experimentally the degree of correlation between the new $L$ value, the $E$ value at 328m$\mu$ and the blue value. For this purpose a series of measurements was made on unsaponifiable residues (except in the case of concentrated sources) to eliminate factors of inhibition which constitute an important (though entirely unnecessary) source of discrepancy between the chemical and physical values. The conversion factors for the above units were determined on the basis of measurements on 21 oils and 18 concentrates as follows:

$E$ (1%, 1 cm., 328m$\mu$) = $L$ value $\times$ (0·41 $\pm$ 0·05).

Blue value = $L$ value $\times$ (20 $\pm$ 3·0).

The ratio of $L$ value to $E$ value at 620m$\mu$ was calculated from equation (1) and was not measured on these oils and concentrates. The calculation showed that $E$ (1%, 1 cm., 620m$\mu$) = $L$ value $\times$ (1·30 $\pm$ 0·03). Bearing in mind the nature and magnitude of the various sources of discrepancy so ably discussed by Morgan *et al.* [1935], we feel that the above conversion factors will yield accurate results (within the limits indicated above) for those who wish to change from one unit to another. The question of the correlation of the $L$ value with the biological assay will be dealt with separately.

## Summary

1. The Evelyn photoelectric colorimeter has been applied to the measurement of the intensity of the blue colour produced by the reaction between vitamin A and antimony trichloride; the simple procedure for the estimation of vitamin A in oils and other materials is described. The method combines accuracy, convenience and economy perhaps to a greater extent than any other.

2. The most important advantage of this new method is its objectivity which ensures that the results obtained are as far as possible independent of the operator, thereby eliminating a source of serious error. Other advantages are rapidity and convenience.

3. Data are presented to illustrate the precision of the method, which is considerably greater than that obtained with the tintometer or the visual spectrophotometer.

4. A new unit is defined for the expression of results obtained by the method, and experimental data are given on the relation between the new unit and other units now in use.

One of us (K. A. E.) is indebted to the Banting Research Foundation, Toronto, Canada, for several grants during the period of development of the colorimeter as well as in support of the present work.

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# CXXXVII. DETERMINATION OF VITAMIN K BY THE CURATIVE BLOOD-CLOTTING METHOD

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(*Received 2 May 1938*)

In this laboratory the determination of vitamin K (the fat-soluble anti-haemorrhagic vitamin) has been carried out during the last 2 years by means of the curative blood-clotting method. This method was originally described by Schønheyder [1936, 1, 2] and has since been subjected to a thorough revision.

The method is based on the procedure and apparatus used by Fischer [1930] in his research on the coagulability of bird's plasma.

Blood is taken from the carotid artery of the chick and centrifuged, and the plasma is diluted with an equal part of Ringer's solution. Four droplets ($4 \times 0{\cdot}05$ ml.) of the diluted plasma are introduced into the micro test tube of Fischer, and one droplet of tissue extract (0·05 ml.) is added. The test tube is held by a metal frame provided with a detachable cover which closes the tube. The frame is placed in a constant temperature water bath (39°) and rotated in order to mix the contents. By rotating the frame four times a minute, the clotting time is easily measured by a stop-watch.

By using a series of different concentrations of the tissue extract it is possible to find the concentration ($K$) which produces clotting in 3 min. If the concentration of extract which clots a normal plasma in 3 min. is $K_n$, the quotient $\frac{K}{K_n}$ ($=R$) will characterize the degree of reduction of the clotting power while the reciprocal value will be a direct measure of the clotting power. Normal chicks have the value $R=1$, with a certain individual variation in both directions.

When the $R$-values ($R_1$) of a series of $K$-avitaminous chicks have been determined the animals receive on three successive days a weighed amount of a test substance in the form of tablets. On the 4th day the $R$-value is determined again ($R_2$). The animals are weighed every day during the 3 days' period and the mean weight is calculated.

From the results of this experiment a diagram can be constructed showing the variation of the $R_2$-values with the amount of test substance ingested (mg. test substance per g. body wt. per day).

From the amount ingested per g. body wt. per day and the $R_2$ value it is possible to calculate, by extrapolation, how much of the substance should be given in order to render the clotting power normal ($R_2=1$), provided that the $R_2$-value is found to be above 1. This amount of the substance is said to contain 1 curative unit.

Our experience with the method has shown us the necessity for introducing certain improvements.

It is necessary to have a constant and uniform tissue extract. The watery extracts of chick's embryo or hen's lung used by Fischer [1930] and by Schønheyder [1936, 1, 2] exert a strong coagulating effect, but are heterogeneous suspensions liable to undergo sedimentation and putrefaction when stored in a flask in the ice box in a liquid state, even when antiseptics are added. These preparations

[1] This work has been aided by a grant from the Josiah Macy, Jr. Foundation and P. Carl Petersens Fond.

act as thrombokinase, but it has been found that different preparations may yield different $R$-values with the same chicken plasma, owing, in all probability, to contamination with thrombin, even if their power of clotting oxalated plasma may not be demonstrable.

A fairly constant extract may be prepared in the following way.

White muscle (200 g.) from the breast of a hen killed by bleeding is minced and ground with an equal weight of pure sand and 200 ml. Ringer's solution (or a similar solution without phosphate and bicarbonate). After suitable centrifuging and filtering through paper a milky liquid is obtained. This liquid (about 200 ml.) is cooled on ice and, under continuous stirring, is put into ampoules, 0·5 ml. being carefully measured into each by means of the syringe pipette of Krogh. The ampoules are closed, frozen in a freezing mixture and stored in the inner box of the frigidaire at approx. 3°. When determinations are to be made the contents of 1 ampoule are drawn up in a syringe and transferred to a 5 ml. measuring flask; the syringe and ampoule are rinsed with Ringer's solution (or the corresponding solution without phosphate and bicarbonate), the contents of the flask being brought up to the mark with the wash liquid. The solution now represents a 10 % concentration of the extract, from which lower concentrations 5, 2·5, 1, 0·5, 0·25, and 0·1 % are prepared.

The calculation of $K$ from the clotting time obtained with different concentrations is facilitated by plotting the clotting times in seconds against the corresponding concentrations on paper ruled with logarithmic axes.

Determinations of $K$ for the plasma from a normal chick, made by a series of dilutions from 10 ampoules, gave the following values:

0·156, 0·153, 0·160, 0·185, 0·188, 0·164, 0·180, 0·180, 0·182, 0·182.

The mean value was 0·173 and the maximum deviation from the mean value was 12 %.

The values of $K$ for another normal plasma, determined at intervals of 30 min. during 4 hr., ranged from 0·22 to 0·25, the highest values being found at the end of the period. This variation was due to the fact that the weakest concentrations—0·05 %—had deteriorated somewhat. The dilutions should therefore preferably be kept at 0° when they are to be used over a period of several hours.

The mean normal value of $K$ was determined for 10 normal chicks by one and the same series of dilutions made from the mixed contents of 3 ampoules. The diet used for the normal chicks was a commercial chicken ration in which the principal source of vitamin K was 4 % of dried alfalfa containing about 300 units of vitamin per g. The following individual values were obtained:

0·175, 0·100, 0·100, 0·124, 0·158, 0·200, 0·170, 0·100, 0·085, 0·157.

The mean value was 0·137 and the maximum deviation from the mean value was 46 %.

This mean value of $K_n$ was used in the calculation of $R$-values, but it is possible that a somewhat higher or lower $K_n$ value might have been found if a much larger number of animals had been used.

The Ringer's solution must always have exactly the same composition; the modified Ringer's solution may consist of NaCl 0·95 %, KCl 0·02 %, $CaCl_2$ 0·02 %. It is advisable to determine the $K_n$ value at intervals.

After storage in the frozen state for several months the tissue extract is rendered flocculent by dilution with Ringer's solution, but the mean value of $K_n$ is not altered.

The diet used to produce the haemorrhagic disease may vary within wide limits. It is difficult to obtain a source of protein which is either free from vitamin K or has a constant low vitamin K content.

The following are some of the diets which have been used:

Diet 124*c*

| | |
|---|---|
| Ether-extracted dried hog liver | 15 |
| Dried yeast | 12 |
| Salts no. 2* | 2 |
| Sucrose | 71 |
| | 100 |
| Cod liver oil | 2 |

* Salts no. 2 consist of $CaCO_3$, 400; $MgCO_3$, 20; NaCl, 88; ferric citrate, 32; KI, trace.

Diet 417

| | |
|---|---|
| Ether extracted dried fish meal | 17·5 |
| Dried yeast | 7·5 |
| Sucrose | 73 |
| Salts* | 1 |
| Cod liver oil | 1 |
| | 100 |

* Salts consist of NaCl, 85; ferric citrate, 14; $CuSO_4$, 1.

Diet 451

| | |
|---|---|
| Casein | 15 |
| Dried yeast | 10 |
| Salts no. 2 | 2 |
| Sucrose | 73 |
| | 100 |
| Cod liver oil | 1 |

Diet 124*c* enables chickens to thrive fairly well, even if it does not contain all the factors necessary for the chick except vitamin K. It is, however, rather expensive.

Diet 417 resembles the diet of Almquist & Stokstad [1936]. Experience has shown that it may be difficult to obtain a fish meal of constantly low vitamin K content, a circumstance which is probably explained by the possibility of K-vitamin formation by putrefaction of the product before drying.

Diet 451 has often given good results, but in some cases the same difficulty of obtaining protein free from vitamin K has been encountered.

Experience shows further that some of the birds receiving these diets develop the encephalomalacia described by Goettsch & Pappenheimer [1936].

It is absolutely necessary to keep the animals clean, i.e. to change and clean the wire screen bottoms once or twice a day and the food and water containers twice. Animals kept in dirty cages are to a large extent protected from the disease and often have practically normal $R$-values even after months on the diet. On the other hand, cleanliness will not ensure that all animals have sufficiently high $R$-values after 3–4 weeks.

In determining his curve Schønheyder [1936, 1, 2] made the supposition that chicks which had received the vitamin K-free diet for at least 3 weeks had a constant $R_1$ value of about 150–200 and he therefore did not determine the $R_1$ value before the ingestion of the test substance. Our own experience, which extends over several years, has shown however that the $R_1$ values may vary widely in different individuals, even when precautions are taken to secure uniformity of the food, cleanliness and a constant tissue extract.

Examples of individual variations are given in the following table:

| Chick no | Diet no. | No. of days on the diet | $R_1$ |
|---|---|---|---|
| 2749 | 124 *c* | 23 | 750 |
| 2734 | 124 *c* | 28 | 7·4 |
| 2738 | 124 *c* | 36 | 96 |
| 2775 | 124 *c* | 25 | 20 |
| 2792 | 124 *c* | 25 | 280 |
| 2802 | 417 | 22 | 280 |
| 2814 | 417 | 24 | 13 |
| 2819 | 417 | 24 | 600 |
| 2829 | 124 *c* | 23 | 29 |
| 2834 | 124 *c* | 24 | 1·3 |

A standard source of vitamin K has been chosen, consisting of a selected and very uniform dried spinach which has been finely powdered and formed into tablets each weighing 0·25 g. The preparation is stored in completely filled containers at +3°. This standard was found by the construction of the curve to contain 500 units per g.

*Construction of the curve (Fig. 1)*

The animals were given the vitamin K-free diet when they weighed 100–130 g. $R_1$ was determined when they showed haemorrhages. The ingestion of the standard substance began on the day when the $R_1$ value was determined, and the determination of the $R_2$ value followed on the day after the third ingestion.

Animals having $R_1$ values between 60 and 140 were used for small quantities of the standard substance, while those with lower $R_1$ values were used for the larger quantities. It is advisable to utilize animals having $R_1$ values from 20 to 60 in order to save cost. Animals having such $R$ values generally survive better than those having the highest values. The curve is drawn on paper with linear abscissae and logarithmic ordinates.

The points corresponding to animals with high $R_1$ values are first plotted and the left-hand part of the curve—corresponding to small amounts of the standard substance and $R_2$ values above 4—is drawn. For those points of the right-hand part of the curve (below $R_2=3$) which have been obtained from animals having $R_1$ values lying between 60 and 20 a correction is added to the abscissa. This correction is taken from the left-hand part of the curve, which does not need any correction, and is equal to that quantity of the standard substance which is required to depress the $R$ value from between 60 to 140 to the lower $R_1$ value in question.

The table on p. 1022 gives a survey of the results.

The diagram shows that about 2 mg. of the standard substance per g. body weight per day are the smallest quantity which will make the clotting power normal ($R=1$). This means that 2 mg. of the standard spinach contain about 1 unit. 1 g. therefore contains 500 units.

Future determinations can now be based on this standard substance; this means that the vitamin content of a substance which has produced a decrease of the $R$ value from $R_1$ to $R_2$ can be calculated from the amount of the standard spinach which on the diagram produces the same decrease of the $R$ value, the spinach being assumed to contain 500 units per g.

The diagram shows that the limits of error are considerable. An exact calculation of the mean error has not been attempted, owing to the unequal distribution of the individual determinations.

| Chick no. | Mean wt. during the 3-day period | Mg. standard substance per g. body wt. per day | $R_1$ | $R_2$ | Corrected abscissa |
|---|---|---|---|---|---|
| 3521 | 241 | 2·9 | 117 | 1·35 | 2·9 |
| 3557 | 367 | 6·8 | 280 | 1·0 | 6·8 |
| 3583 | 296 | 12·8 | 100 | 1·0 | 12·8 |
| 3576 | 405 | 3·16 | 130 | 0·76 | 3·16 |
| 3572 | 413 | 1·82 | 42 | 1·1 | 1·86 |
| 3670 | 225 | 1·11 | 39 | 1·83 | 1·15 |
| 3580 | 257 | 4·28 | 23 | 0·66 | 4·37 |
| 3666 | 300 | 0·25 | 150 | 10·0 | 0·25 |
| 3695 | 442 | 1·36 | 55 | 0·79 | 1·38 |
| 3718 | 393 | 4·07 | 31 | 0·73 | 4·13 |
| 3672 | 390 | 0·77 | 62 | 2·15 | 0·77 |
| 3701 | 390 | 0·205 | 100 | 12·8 | 0·205 |
| 3713 | 250 | 4·00 | 22 | 1·8 | 4·1 |
| 3738 | 266 | 0·43 | 73 | 4·66 | 0·43 |
| 3671 | 480 | 0·104 | 86 | 16·0 | 0·104 |
| 3727 | 360 | 0·97 | 62 | 1·67 | 0·97 |
| 3698 | 423 | 0·59 | 88 | 1·5 | 0·59 |
| 3722 | 375 | 1·33 | 40 | 1·63 | 1·37 |
| 3885 | 333 | 0·192 | 100 | 7·3 | 0·192 |
| 3577 | 394 | 1·90 | 39 | 1·34 | 1·94 |
| 3610 | 290 | 0·516 | 145 | 2·00 | 0·516 |

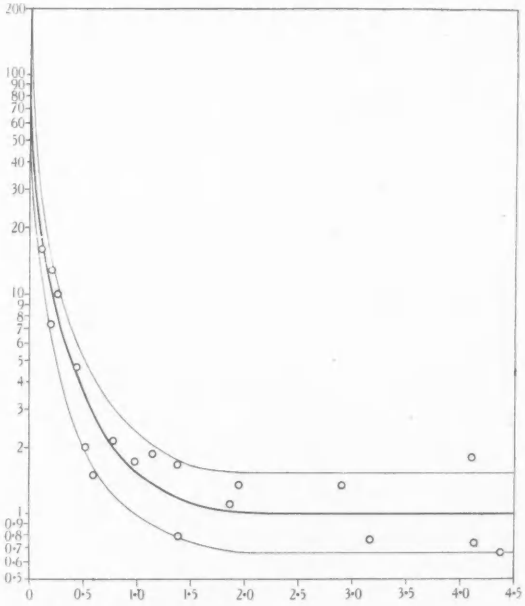


Fig. 1. Abscissa: mg. standard substance per g. body weight per day. Ordinate: $R$-value.

Inspection of the area containing the different points shows that the maximum horizontal deviation from the curve is about $\pm 40\,\%$ for $R_2$ values between 20 and 2. For animals having high $R_1$ values (60–140) the error of one single determination appears to be of that order, but if the $R_1$ value is low, the error is

correspondingly increased. $R_2$ values lying below 1·8 may only give minimum values for the strength of the preparations. It is therefore necessary to make more than one determination when an accurate estimation is to be made.

The error may be enormous if the $R_1$ values are not determined even if $R_2$ is greater than 2.

It is further noted that the variation of the ordinates corresponding to a given abscissa is not due to the variation of the $R_1$ values. Animals with a high $R_1$ value may show $R_2$ values lying below the curve and vice versa.

The diagram shows further the important fact that it is not possible to render the coagulability of the plasma "supernormal" by ingesting large doses of the vitamin. The $R$ values obtained by giving more than 1 unit per g. are distributed about the line $R=1$.


## Summary

The determination of vitamin K by the curative blood-clotting method has been subjected to a thorough revision.

A tissue extract of constant activity was made by putting up an extract of hen's muscle in ampoules and storing it in a frozen condition.

The activity of the tissue extract was measured against the plasma of a series of normal chicks.

A selected dried spinach powder formed into tablets and stored under precautions securing stability was chosen as a standard of vitamin K. This standard was used for the construction of a curve of reference; it was found to contain 500 units per g.

The limits of error are discussed.

It was found to be impossible to render the coagulability of the blood plasma "supernormal" by administering large doses of the standard substance.


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# CXXXVIII. HEPATIC OXIDATION OF CHOLINE AND ARSENOCHOLINE

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(*Received 30 April 1938*)

It was shown by Mann & Quastel [1937] that choline is oxidized by slices or extracts of rat liver and kidney. The oxidation product was isolated and identified as betaine aldehyde. Some evidence was obtained that betaine aldehyde is also oxidized by the same tissues though at a much slower rate than choline and it was suggested that the oxidation product in this case is betaine. Bernheim & Bernheim [1933] observed an increase in oxygen uptake when acetylcholine was added to extracts of liver and kidney of rat and cat and suggested that it was due to choline oxidation. The same authors in 1938 [cf. also Bernheim & Webster, 1937] arrived at the conclusion that betaine aldehyde is formed, without, however, isolating it. They showed that synthetic betaine aldehyde is oxidized by liver extract, the rate of oxidation being dependent upon *p*H. At *p*H 7·8 it is oxidized presumably to betaine but at *p*H 6·7 the aldehyde suffers little change.

In the present work it is shown that one of the components of the choline-oxidizing system is a typical dehydrogenase which can bring about the oxidation of choline anaerobically, provided that a suitable hydrogen acceptor is present, and which is insensitive to low concentrations of cyanide though the complete system is cyanide-sensitive. Some evidence is also put forward which indicates that the other components of the oxidizing system are cytochrome and indophenol oxidase. The oxidation of arsenocholine by the same system is also described.

## *Methods*

Choline and arsenocholine[1] were both used as the chlorides. The enzyme preparations employed were made from rat liver by the method already described by Mann & Quastel [1937]. For the anaerobic experiments with ferricyanide which were carried out in bicarbonate buffer a dialysed preparation made according to the description of Bernheim & Bernheim [1938] was employed. All manometric experiments were carried out in the Warburg apparatus at 37°, the total volume of medium in each vessel being always 3·0 ml. Aerobic experiments were carried out in a sodium phosphate buffer medium of final concentration 0·06 *M* at *p*H 7·4 in air. Anaerobic experiments were carried out in 0·025 *M* bicarbonate in an atmosphere of 95% $N_2$+5% $CO_2$. Sodium ferricyanide was used in these experiments as the hydrogen acceptor according to Quastel & Wheatley [1938], and the oxidation was followed by measuring the $CO_2$ output.

[1] We are much indebted to Dr Welch and to Messrs Hoffmann-la Roche and Co., Ltd., for their kindness in presenting us with specimens of arsenocholine chloride.

*The oxidation of arsenocholine*

The effect of addition of arsenocholine on the $O_2$ uptake of rat liver extract is shown by the curve of Fig. 1. The required amounts of arsenocholine chloride solution were placed in the side tubes of the Warburg vessels and tipped into the main compartment immediately after the initial readings had been taken. In the figure the values of the $O_2$ uptakes in the first 15 min. are plotted against the amount of arsenocholine chloride present. It will be observed that the optimum concentration of substrate is not reached even with 40 mg. arsenocholine chloride in a total volume of 3 ml. (i.e. 0·07 *M*). With choline chloride, as has previously been shown, the optimum concentration is of the order of 1 mg. per ml. (i.e. 0·007 *M*). Hence the enzyme has a comparatively low affinity for arsenocholine.

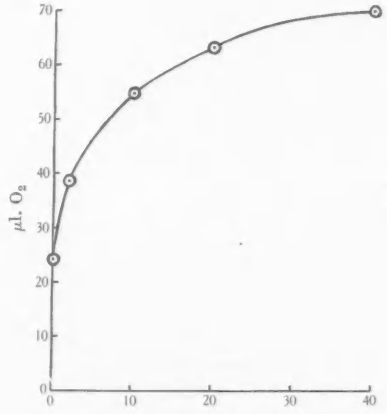



Fig. 1.

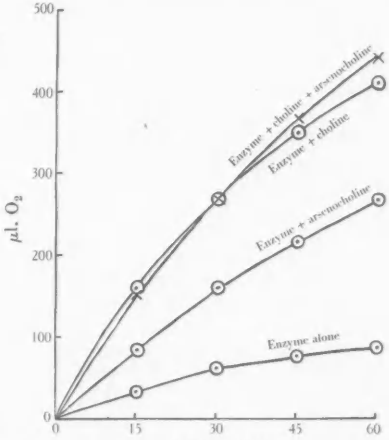



Fig. 2.

Fig. 1. The effect of addition of arsenocholine on the $O_2$ uptake of rat liver extract.

Fig. 2. The effect of 5 mg. choline chloride, 5 mg. arsenocholine chloride and a mixture of 5 mg. arsenocholine chloride + 5 mg. choline chloride on the $O_2$ uptake of the liver extract.

That the dehydrogenase concerned in the oxidation of arsenocholine is identical with choline dehydrogenase is indicated by the fact that competition occurs between choline and arsenocholine as substrates. This is shown by the curves of Fig. 2 where the $O_2$ uptakes produced by the addition of 5 mg. choline chloride, 5 mg. arsenocholine chloride and by a mixture of 5 mg. arsenocholine chloride + 5 mg. choline chloride to the liver extract are plotted. Under these initial conditions the additional $O_2$ uptake produced by choline is rather more than double that produced by arsenocholine. The initial concentration of choline is sufficient to saturate the enzyme. When choline and arsenocholine are both present the $O_2$ uptake is not additive as would be expected if the substrates were activated by different enzymes but on the contrary is slightly less than when choline alone is present. The diminution is small owing to the much greater affinity of the enzyme for choline than for arsenocholine. As oxidation proceeds the residual choline becomes insufficient to saturate the enzyme and hence the

uptake becomes higher where both choline and arsenocholine are present together.

Judging from the total $O_2$ uptake the oxidation of arsenocholine follows a course analogous to that of choline. The total extra $O_2$ uptake per mg. arsenocholine chloride added is in the neighbourhood of the 112 $\mu$l. necessary if 1 mol. of arsenocholine requires 1 mol. of $O_2$. This would suggest that the final product of oxidation is arsenobetaine. Thus in the experiment illustrated by the curves of Fig. 3 the value is 107 $\mu$l. Furthermore in the early stages of the oxidation a substance is formed which reacts with 2:4-dinitrophenylhydrazine. This may be conveniently demonstrated by adding to 1 ml. of the reaction mixture 1 ml. of a saturated solution of 2:4-dinitrophenylhydrazine in *N* HCl, keeping the mixture at room temperature for 30 min. and making alkaline by addition of 2·5 ml. *N* NaOH when a deep red colour is produced indicating the presence of a hydrazone. The substance reacting with the phenylhydrazine is presumably

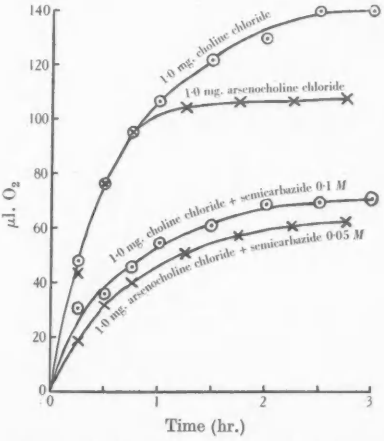



Fig. 3. The effect of semicarbazide on the oxidation of choline and arsenocholine.

arsenobetaine aldehyde. During the later stages of the oxidation the reaction with 2:4-dinitrophenylhydrazine diminishes in intensity and finally becomes no greater than that of the control without added arsenocholine. It appears therefore that, as with choline, a comparatively rapid oxidation to the aldehyde occurs, the latter being oxidized at a much slower rate to the corresponding acid. It is evident however that in the case of arsenocholine a secondary reaction takes place. During the oxidation a strong garlic odour suggestive of trimethylarsine is produced, and acid permanganate placed in the side tubes of the Warburg vessels is decoloured, indicating the formation of a volatile reducing substance.

*Use of semicarbazide as fixative*

The oxidations both of choline and of arsenocholine may be arrested at the aldehyde stage if semicarbazide is used as a fixative. This is shown by the curves of Fig. 3 which illustrate the course of oxidation of choline and arsenocholine in presence and absence of semicarbazide. The concentration of semicarbazide used was 0·1 *M* in the choline experiment and 0·05 *M* in the arsenocholine

experiment. The amount of substrate added in each case was 1 mg. of the chloride. The control figures for the $O_2$ uptake of the enzyme preparation in presence and absence of semicarbazide have been subtracted and the extra $O_2$ uptake due to the added substrate is plotted. The experiments with choline and arsenocholine were carried out on different days and have been fitted on to the same figure for convenience. It will be observed that in presence of semicarbazide the $O_2$ uptakes with both substrates correspond roughly with the theoretical value assuming that the substrate molecule takes up 1 atom of oxygen. In absence of semicarbazide however the $O_2$ uptake attains values nearly equivalent to 1 mol. $O_2$ per mol. of substrate. Thus in presence of semicarbazide the actual figures obtained are 71 and 62 $\mu$l. for choline and arsenocholine respectively, whereas the theoretical figures are 80 and 56 $\mu$l. In absence of semicarbazide the figures obtained are 140 and 107 $\mu$l. and the theoretical figures 160 and 112 $\mu$l. Dinitrophenylhydrazine tests carried out at the conclusion of the experiments in the manner already described give strongly positive results where semicarbazide has been present and weak or negative ones in its absence. This indicates that the effect of semicarbazide is due to fixation of betaine aldehyde and arsenobetaine aldehyde.

It has already been reported that during the oxidation of arsenocholine volatile reducing substances are formed, as shown by the reduction of acid permanganate placed in the side tubes of the manometric vessels. If the oxidation is carried out in presence of semicarbazide this reduction of acid permanganate does not occur. Either the secondary reaction does not take place in presence of semicarbazide or the products of this reaction also are bound by semicarbazide. Attempts to isolate arsenobetaine aldehyde from the reaction mixture have so far only yielded mixtures and it is evident that a secondary decomposition takes place. In the case of choline oxidation however where apparently no secondary reaction occurs it should be possible to simplify the method used by Mann & Quastel [1937] for separation of betaine aldehyde either by the use of semicarbazide as a fixative or by utilizing the finding of Bernheim & Bernheim [1938] that at *p*H 6·7 the oxidation of betaine aldehyde proceeds only very slowly.

*Reduction of ferricyanide*

The properties of choline dehydrogenase may be conveniently studied by the use of ferricyanide as a hydrogen acceptor as shown by Quastel & Wheatley [1938]. The experiments are carried out in a bicarbonate (0·025 *M*) medium in an atmosphere of 95% $N_2$+5% $CO_2$, and the reaction is followed by measuring the $CO_2$ displaced from the bicarbonate by the acid produced by reduction of the ferricyanide according to the equations

$$H + Fe(CN)_6''' \rightarrow H^+ + Fe(CN)_6'''',$$
$$H^+ + HCO_3' \rightarrow H_2CO_3 \rightarrow CO_2 + H_2O.$$

A typical curve illustrating the oxidation of choline by this method is given in Fig. 4. The amount of choline chloride used in this experiment was 0·5 mg. A control vessel containing 0·5 mg. choline chloride and ferricyanide but no enzyme preparation showed a $CO_2$ output of only 9·4 $\mu$l. during the experimental period.

Bernheim & Bernheim [1938] report that cyanide in fairly large concentrations inhibits the oxidation of choline but that the inhibition is of rather short duration. This is found not to be the case when the inner cups of the manometer vessels contain the appropriate potash-cyanide mixture to prevent

change in the concentration of cyanide in the reaction mixture during the course of the experiment [Mann & Quastel, 1937]. In anaerobic experiments however using ferricyanide as the hydrogen acceptor concentrations of cyanide which produce almost complete inhibition of choline oxidation aerobically here show little or no inhibition. The effects of cyanide aerobically and anaerobically

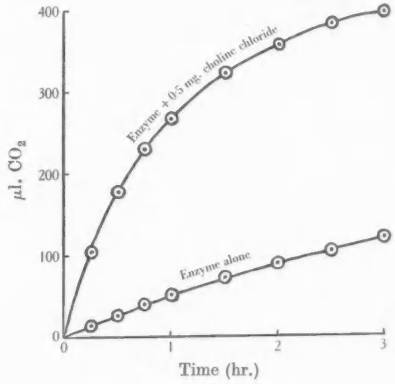



Fig. 4. The anaerobic oxidation of choline with ferricyanide as a hydrogen acceptor.

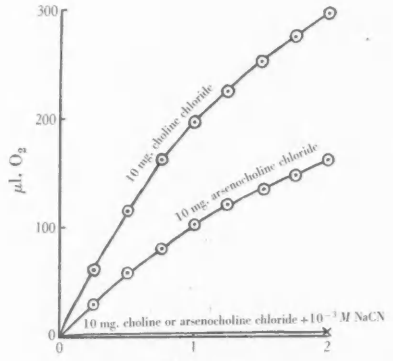



Fig. 5.

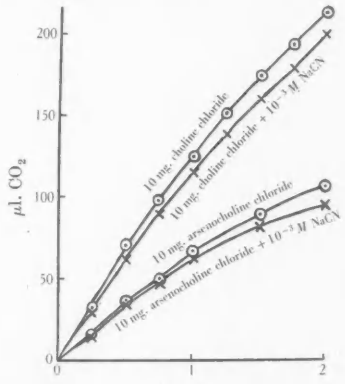



Fig. 6.

Fig. 5. The complete inhibition of aerobic oxidation of choline and arsenocholine by $10^{-3}$ *M* NaCN.

Fig. 6. The effect of $10^{-3}$ *M* NaCN on the anaerobic oxidation of choline and arsenocholine.

on choline and arsenocholine oxidation are illustrated by the curves of Figs. 5 and 6. Sodium cyanide was used in both aerobic and anaerobic experiments at a final concentration $10^{-3}M$. The amount of choline and arsenocholine chloride added to each vessel was 10 mg. The control figures for the $CO_2$ output or $O_2$ uptake of the enzyme preparation alone and in presence of cyanide have been

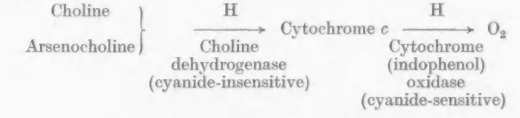
subtracted from the values obtained in presence of choline and arsenocholine. Since the inhibitory effect of cyanide is exercised aerobically but not anaerobically it is apparent that the component of the oxidation system which is cyanide-sensitive is not the choline dehydrogenase. The latter is comparatively cyanide-insensitive though results of experiments with higher concentrations of cyanide indicate some sensitivity to concentrations of the order of $10^{-1}M$.

*Reduction of cytochrome* c

Choline dehydrogenase in presence of either choline or arsenocholine brings about a rapid reduction of cytochrome *c*.[1] This has been established by anaerobic experiments carried out in Thunberg tubes, the reduced cytochrome being identified by its absorption spectrum by means of the microspectroscope. One such experiment may be described here.

A rat liver was chopped with scissors, washed repeatedly with tap water by decantation, and the residue was ground in a mortar with sand and 10 ml. 0·1 *M* phosphate buffer *p*H 7·4. The mixture was squeezed through muslin and the resulting suspension was centrifuged. The precipitate was mixed with water, again centrifuged, and finally suspended in 0·1 *M* phosphate buffer *p*H 7·4. An amount of the suspension equivalent to 0·1 g. liver was placed in each of three Thunberg tubes. One tube contained no added substrate, the second contained 10 mg. choline chloride and the third 10 mg. arsenocholine chloride. To each tube 0·15 ml. of a solution of cytochrome *c* (5 mg. per ml.) was added and the total volume was made up to 3 ml. in each case. On evacuation at room temperature the tubes containing choline and arsenocholine showed the spectrum of reduced cytochrome *c* at once. A strong band was seen at about 550 m$\mu$. and a weak one at about 520 m$\mu$. On shaking the suspensions with air the bands of reduced cytochrome quickly disappeared but reappeared in a few seconds when the tubes were again evacuated. Neither the enzyme preparation alone nor the heat-inactivated enzyme in presence of choline showed any reduction of cytochrome *c* under anaerobic conditions in Thunberg tubes after 15 min. at room temperature. Guinea-pig liver preparations made in a similar manner which do not oxidize choline [Mann & Quastel, 1937] showed no reduction of cytochrome *c* in presence of choline, though a rapid reduction was obtained in presence of succinate.

These results make it evident that choline dehydrogenase in presence of its substrates, choline or arsenocholine, can accomplish the reduction of cytochrome *c*, and there is every reason to believe therefore that the complete choline oxidase system consists of choline dehydrogenase, cytochrome *c* and cytochrome oxidase (indophenol oxidase). Thus the transfer of hydrogen may be expressed by the scheme:




Such a scheme accounts for the cyanide-insensitivity of the anaerobic oxidation of choline or arsenocholine by ferricyanide and the cyanide-sensitivity of the oxidation by oxygen. Whether the participation of a carrier or coenzyme is necessary between choline dehydrogenase and cytochrome is not known but there is no evidence for this so far. Choline dehydrogenase, like succinic acid

[1] We are much indebted to Prof. Keilin for a specimen of cytochrome *c*.

dehydrogenase in tissue extracts reduces ferricyanide rapidly, whereas, as shown by Quastel & Wheatley [1938], the rapid reduction of ferricyanide by lactate, *l* malate etc., under similar conditions, requires the addition of cozymase. Prolonged dialysis of the enzyme preparation has little effect on the activity and addition of cozymase after such treatment does not lead to any increase in the rate of oxidation. Attempts to separate the dehydrogenase from cytochrome and then to reconstitute the complete system by addition of cytochrome have as yet been unsuccessful. This is doubtless due to the unusually great instability of the dehydrogenase. The routine methods of preparation, such as precipitation of tissue extracts with acetone or ammonium sulphate, or by acidification, apparently all cause complete destruction of the dehydrogenase.

*Some properties of choline dehydrogenase*

The conclusion that choline dehydrogenase activates both arsenocholine and choline is borne out not only by the fact that competition takes place between the substrates for the enzyme but by the fact that tissue extracts which fail to oxidase choline also fail to oxidase arsenocholine. For example, a guinea-pig liver extract which took up 36·3 $\mu$l. $O_2$ in 1 hr. at 37° took up 36·2 $\mu$l. under the same conditions in presence of 20 mg. arsenocholine chloride.

Choline dehydrogenase is distinct from alcohol dehydrogenase. Liver extracts, prepared for the experiments cited in this paper, oxidize both choline and alcohol. When a mixture of these two substrates is present the $O_2$ uptake is additive. This is shown by the following figures:

| | | $\mu$l. |
|---|---|---|
| $O_2$ uptake by rat liver extract alone in 45 min. at 37° | | 64·9 |
| ,, ,, | +choline (10 mg.) | 283·7 |
| ,, ,, | +alcohol (5 mg.) | 114·1 |
| ,, ,, | +choline (10 mg.) and alcohol (5 mg.) | 332·7 |
| The theoretical figure for additive oxygen uptakes | | =332·9 |

Bernheim & Bernheim [1938] showed that a dialysed preparation of choline oxidase is unable to oxidize alcohol, and conversely that an aqueous extract of acetone-dried liver oxidizes alcohol but not choline. Clearly the $-CH_2OH$ group is not a competing group for the enzyme. On the other hand, the group $>NR_3$ ($R$=H or $CH_3$) seems to be of primary importance for the attachment of the substrate to the enzyme. This is shown by the fact that $NH_4^+$, $NH(CH_3)_3^+$, and betaine inhibit choline oxidation. Typical curves illustrating the inhibitory activities of these substances are shown in Figs. 7, 8 and 9. It will be observed that ammonium chloride decreases the $O_2$ uptake of the liver extract alone. This is possibly due to inhibition of oxidation of choline. Access to the enzyme would appear to depend on the presence of the $>NR_3$ group in the substrate.

As is evident from the oxidation of arsenocholine by choline dehydrogenase, the N atom of the grouping in question may be replaced by As, with loss, however, in affinity for the enzyme. The Michaelis constant of the enzyme for choline is about 0·0012 $M$ and for arsenocholine apparently about 0·008 $M$.

It is of interest that, as shown by Roepke [1937], acetylarsenocholine is hydrolysed by choline esterase and has an affinity for that enzyme as great as that of acetylcholine. Both choline and arsenocholine inhibit choline esterase, both substrates having approximately equal affinities. Thus substitution of N by As still clearly allows accessibility to the enzyme to take place.

## Discussion

The evidence presented in this paper shows that choline and arsenocholine are both oxidized by the same enzyme system to the respective aldehydes. The enzyme system in question consists partly of a typical dehydrogenase and partly

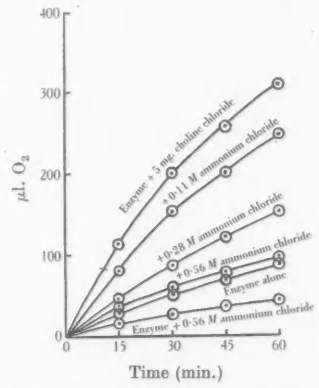



Fig. 7. Inhibition of choline oxidation by ammonium chloride.

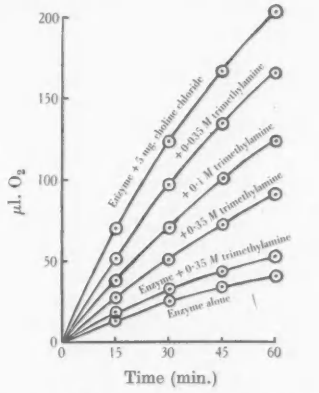



Fig. 8. Inhibition of choline oxidation by trimethylamine.

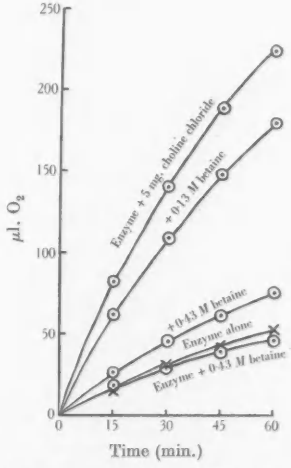



Fig. 9. Inhibition of choline oxidation by betaine.

of the cytochrome-indophenol oxidase system. Cytochrome *c* undergoes reduction by choline or arsenocholine in presence of the dehydrogenase and there is as yet no evidence that a coenzyme takes part in this reduction. It appears therefore that choline dehydrogenase, together with its substrate, like succinic acid dehydrogenase, reduces cytochrome directly. It must be concluded that, in organs in which choline oxidation takes place, that part of the respiration

which includes choline combustion proceeds through the intermediate action of cytochrome. That the enzyme activating choline or arsenocholine is a typical dehydrogenase is shown conclusively by the rapid reduction of ferricyanide which takes place under anaerobic conditions and which is not cyanide-sensitive.

It is of interest that arsenocholine is oxidized by choline dehydrogenase although the substitution of N by As has obviously lowered the affinity of substrate for the enzyme. The grouping $>NR_3$ ($R$=H or $CH_3$) seems to be primarily important for access of substrate to the enzyme as shown by the inhibitions effected by ammonium and trimethylammonium ions and by betaine.

## Summary

1. Arsenocholine, like choline, is oxidized by choline oxidase in rat liver to the corresponding aldehyde. This is followed probably by oxidation to arsenobetaine. The substitution of N by As reduces the affinity of the substrate for the enzyme.

2. Secondary decomposition of arsenocholine takes place in presence of rat liver with liberation of a volatile reducing compound having a strong garlic odour (? trimethylarsine).

3. In presence of semicarbazide, fixation of betaine aldehyde, and arsenobetaine aldehyde, takes place, kinetic studies showing an $O_2$ uptake corresponding to the formation of these substances.

4. Choline oxidase consists partly of a typical dehydrogenase. This is shown by the fact that rat liver extracts, in presence of choline or arsenocholine, rapidly reduce sodium ferricyanide under anaerobic conditions. This system is cyanide-insensitive and may be studied manometrically in a bicarbonate medium.

5. Choline dehydrogenase, in presence of choline or arsenocholine, rapidly reduces cytochrome *c* at room temperature. There is no evidence that a coenzyme is required for the reduction of cytochrome by choline dehydrogenase in presence of its substrates.

It is inferred that the choline oxidase system consists of choline dehydrogenase, cytochrome *c* and cytochrome (indophenol) oxidase.

6. Choline oxidation is inhibited by ammonium ions, trimethylammonium ions and by betaine. It is concluded that the group $>NR_3$ ($R$=H or $CH_3$) is primarily important for the access of the substrate to the enzyme.

One of us (P. J. G. M.) is indebted to the Medical Research Council for a whole time assistance grant and we would like to express our thanks to this same body for a grant towards the equipment of this laboratory.

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# CXXXIX. α-HYDROXYACETOACETIC ACID

## I. PREPARATION, PROPERTIES AND ESTIMATION

## II. METABOLISM IN ANIMAL TISSUES

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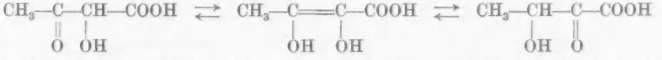
(*Received 2 May 1938*)

## I. PREPARATION, PROPERTIES AND ESTIMATION

α-HYDROXYACETOACETIC acid has aroused the interest of biochemists for two reasons: first, because it was assumed to be a possible intermediate in the oxidative breakdown of acetoacetic acid. Clutterbuck & Raper [1926] studied the oxidation of sodium acetoacetic ester by 1 mol. $H_2O_2$ in the cold and obtained a solution containing a large variety of oxidation and condensation products. Amongst these there was an acid with strong reducing properties which spontaneously decomposed into $CO_2$ and acetol and which they therefore considered to be α-hydroxyacetoacetic acid.

The second reason was the possible existence of the following tautomeric equilibrium between the two ketohydroxy- and the enediol-forms:



α-Hydroxyacetoacetic acid  Dihydroxycrotonic acid  α-Keto-β-hydroxybutyric acid

The enediol tautomeride will share with the other known enediol compounds (which have also been called "reductones") a strong tendency to be dehydrogenated to the diketo-compound. Karrer & Hershberg [1934], in an attempt to prepare these substances, reduced diketobutyric ester and obtained three fractions which they considered to be the ethyl esters of the three tautomerides.

A method is described below for the preparation of solutions containing, in addition to sodium *dl*-α-hydroxyacetoacetate, only an equimolecular quantity of sodium acetate and a little phosphate buffer, and which are therefore suitable for the investigation of the chemical and physiological properties of this substance. The solutions were obtained by hydrolysis of ethyl α-acetoxyacetoacetate [Dimroth & Schweizer, 1923]; these authors have already observed that the ester easily consumes alkali and that it is decomposed to acetol, acetic acid and $CO_2$ in acid solution. They have not however studied the hydrolysis in detail.

On acid hydrolysis an almost immediate evolution of $CO_2$ sets in, whilst on alkaline hydrolysis in air an absorption of $O_2$ occurs. If the hydrolysis is carried out in dilute NaOH under anaerobic conditions, however, and the excess alkali is neutralized before admission of $O_2$, the solution thus obtained shows no appreciable absorption of $O_2$ or evolution of $CO_2$. Hydrolysis is complete at 25° after about 1 hr., when 2 mol. of NaOH have been consumed (Fig. 1).

0·01 ml. or 12 mg. ethyl acetylhydroxyacetoacetate (=0·064 mMol.) used 1·28 ml. $N/10$ NaOH. Calculated (for 2 mol. NaOH per mol. ester): 1·28 ml. $N/10$ NaOH.

The hydrolysis curve was determined in a series of Warburg manometers with two side bulbs, filled with pure $N_2$. At $t_0$ the ester (0·01 ml.) was tipped from one side bulb into 3·0 ml. $N/20$ NaOH. After the time $t$ a measured excess of HCl was tipped from the other side bulb, the vessel quickly disconnected and at once titrated with NaOH against phenolphthalein.

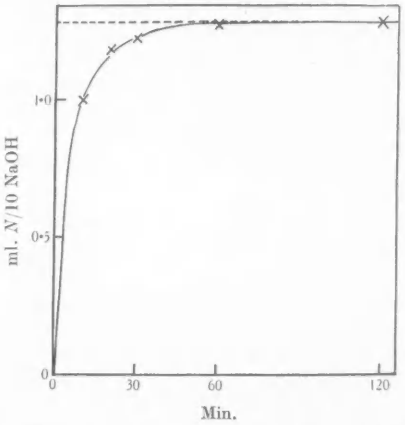



Fig. 1. Hydrolysis of α-acetoxyacetoacetic ester in absence of $O_2$. The broken line indicates the theoretical value for 2 mol. NaOH.

The yield of hydroxyacetoacetic acid depends greatly on the initial concentration of NaOH. If this does not exceed $N/10$, the yield is about 90%, but it declines with higher initial concentrations, probably owing to polymerization (Table I). The final solution is freed from alcohol and concentrated by distillation *in vacuo*.

*Preparation of M/10 sodium hydroxyacetoacetate:* 0·2 ml. ethyl α-acetoxyacetoacetate is added to 26 ml. $N/10$ NaOH in an atmosphere of pure $N_2$. After incubation for 1 hr. at 25° 0·5 ml. $M$ $NaH_2PO_4$ is added in a stream of $N_2$. The solution is concentrated *in vacuo* from a bath at 30° to about 10 ml.

Table I. *Yield of hydroxyacetoacetic acid after hydrolysis of ethyl acetoxyacetoacetate in varying initial concentrations of NaOH. Aniline method*

| Initial concentration of NaOH ($N$) | Yield % |
|---|---|
| 0·1 | 87 |
| 0·2 | 81 |
| 0·37 | 60 |
| 0·87 | 40 |

Whereas the ester linkage of ethyl acetoxyacetoacetate is not hydrolysed in dilute $NaHCO_3$ or only very slowly, the hydrolysis of the acetyl group is accomplished with great ease. This process can be followed manometrically and, in absence of $O_2$, 1 mol. of acid is formed (Fig. 4, curve I). By anaerobic incubation in $NaHCO_3$ α-hydroxyacetoacetic ester can thus be prepared from ethyl α-acetoxyacetoacetate.

0·01 ml. or 12 mg. ethyl acetoxyacetoacetate liberated 1450 μl. $CO_2$ from $N/10$ $NaHCO_3$ in pure $N_2$. Calculated (for 1 mol. of $CO_2$ per mol. of ester): 1438 μl. Warburg manometers filled with Clerici solution were used for these experiments.

*Properties of the solutions obtained by hydrolysis:* the solutions of sodium hydroxyacetoacetate are colourless and odourless. If the hydrolysis has been carried out in an initial concentration of $NaOH > N/10$ they have a distinct yellow colour.

*Colour reactions:* the solutions give a blue colour with $FeCl_3$ and an orange colour with nitroprusside in $NH_3$-alkaline solution. Characteristic colours are obtained with phenols and conc. $H_2SO_4$: with orcin a green colour, with resorcinol a purple, changing to violet and blue, with pyrogallol blue-green, with phloroglucinol dark green.

*Reaction with dinitrophenylhydrazine:* on addition of excess 2:4-dinitrophenylhydrazine a brown precipitate of methylglyoxal-bis-2:4-dinitrophenylhydrazone appears (M.P. 296°), insoluble in $NaHCO_3$ and alcohol, soluble in alcoholic KOH with intense violet-blue colour.

The solutions are free from acetoacetic acid and acetone.

In discussing the hydrolysis of ethyl α-acetoxyacetoacetate reference has been made to three reactions: decarboxylation, autoxidation and polymerization. These will now be dealt with separately.

*Decarboxylation:* hydroxyacetoacetic acid is fairly stable at neutral reaction and low temperature. Thus at *p*H 7·4 the times for 50% decarboxylation are: 6 hr. at 37·5°, 24 hr. at 25° and about 600 hr. at 0°. No difference was found

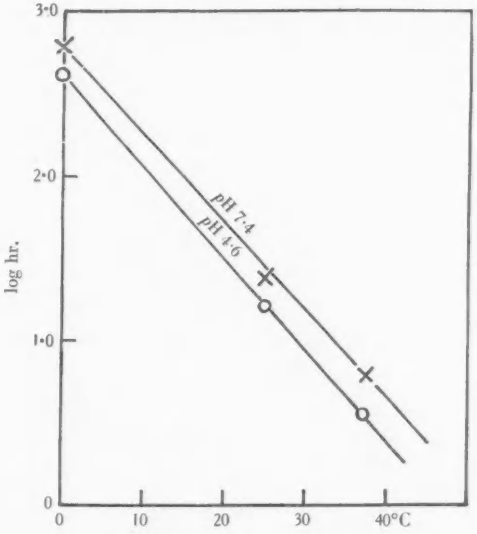



Fig. 2. Half-life time of hydroxyacetoacetic acid as a function of temperature.

between a bicarbonate/$CO_2$ buffer and a phosphate buffer. At *p*H 4·6 the half-life time is only about two-thirds of that at *p*H 7·4 (Fig. 2).

Higher temperatures and lower *p*H greatly accelerate the decarboxylation. At 100° the acid is decarboxylated in a few minutes. In $N/2$ $H_2SO_4$ at 25° the decarboxylation is complete in 90 min. (Fig. 3).

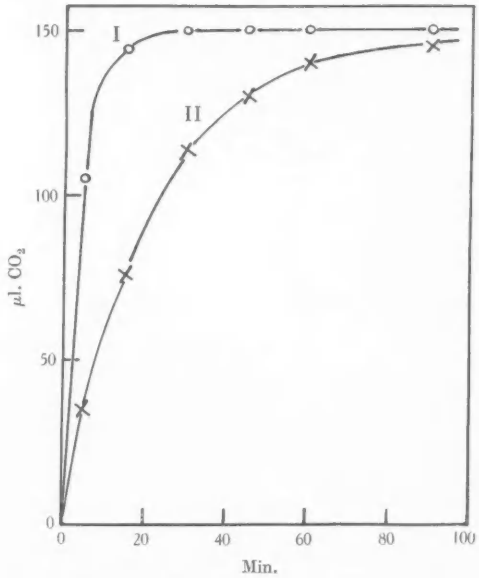



Fig. 3. Decarboxylation of hydroxyacetoacetic acid. I, in presence of aniline; II, in 0·5 $N$ $H_2SO_4$.

The relative stability of the acid at *p*H 4·6 and 25° allows the manometric estimation of bicarbonate in presence of hydroxyacetoacetic acid by acidification with strong acetate buffer *p*H 4·6.

The decarboxylation of hydroxyacetoacetic acid is, like that of other β-ketonic acids, catalysed by aniline [Ljunggren, 1925; Ostern, 1933; Edson, 1935]. In absence of other β-ketonic acids this is the most convenient method of estimation. The solution is brought to *p*H 4·6 with 1/10 vol. of 3 $M$ acetate buffer before the aniline (aniline citrate reagent according to Edson [1935]) is added. The $CO_2$ evolved is measured manometrically at 25°. The decarboxylation is complete in 20 min. (Fig. 3). The decarboxylated product is acetol which can be detected by the very sensitive and apparently specific fluorescence reaction of Baudisch [1918], based on the formation of 3-hydroxyquinaldine by condensation with *o*-aminobenzaldehyde.

*Autoxidation:* (1) *Autoxidation of ethyl hydroxyacetoacetate.* Whereas in absence of $O_2$ only 1 mol. acid is formed from α-acetoxyacetoacetic acid in $N/10$ $NaHCO_3$, due to the hydrolysis of the acetyl group, in presence of $O_2$ 1 mol. $O_2$ is absorbed and 3 mol. acid are formed (Fig. 4).

Three Warburg manometers filled with Clerici solution were used. They all contained ml. $N/10$ $NaHCO_3$ in the main part and 0·008 ml. ethyl acetoxyacetoacetate in the side bulb. Manometer I was filled with pure $N_2$ and served for the estimation of acid formation during

anaerobic hydrolysis. Manometers II and III contained air. 0·2 ml. 10% NaOH was placed in the centre well of manometer vessel II. $O_2$ absorption and $CO_2$ evolution during aerobic hydrolysis were calculated from the pressures observed in manometers II and III. At the end of the experiment (after 6 hr.) an aliquot was taken from the solution in vessel III and its bicarbonate content was determined manometrically. The decrease of $NaHCO_3$ corresponded closely to the $CO_2$ evolution which was therefore due to acid formation and not to decarboxylation.

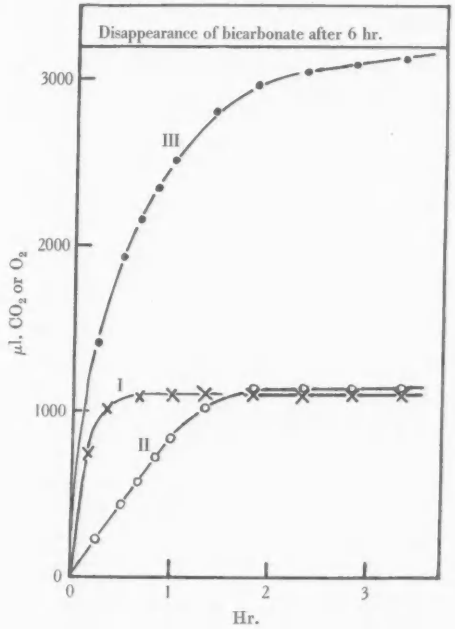



Fig. 4. Hydrolysis and autoxidation of ethyl α-acetoxyacetoacetate in $N/10$ $NaHCO_3$. I, $CO_2$ evolution in pure $N_2$; II, $O_2$ absorption in air; III, $CO_2$ evolution in air.

The liberation of 3 mol. $CO_2$ from $NaHCO_3$ during aerobic hydrolysis of ethyl acetoxyacetoacetate is due to the formation of 2 mol. acetic acid and 1 mol. ethyl hydrogen oxalate. These are found in the solution in theoretical quantity (the latter as oxalic acid after acid hydrolysis).

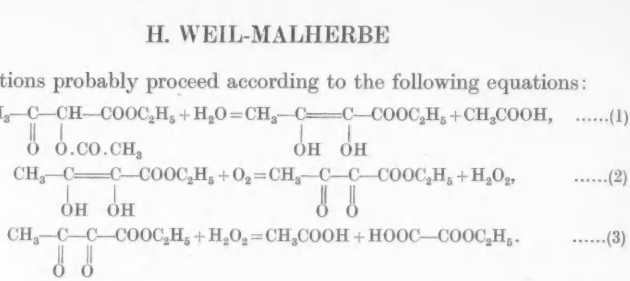
0·008 ml. (=1150 μl.)[1] ethyl α-acetoxyacetoacetate yielded after anaerobic hydrolysis: 1120 μl. volatile acid (estimated as described below); after aerobic hydrolysis: 2160 μl. volatile acid. No formic acid was found.

For the estimation of oxalic acid the solution after acid hydrolysis for 5 min. at 100° was adjusted to *p*H 5 with dilute $NH_3$, an excess of $CaCl_2$ was added and the precipitate centrifuged after 12 hr., washed with $NH_3$ 1 : 50, dissolved in hot $N$ $H_2SO_4$ and titrated with $N/50$ $KMnO_4$ at 70°.

Oxalic acid found after anaerobic hydrolysis: 0; after aerobic hydrolysis: 1080 μl.

[1] Analytical figures are expressed in μl. (1 mMol. of compound in gaseous form at N.T.P. =22,400 μl.) in order to facilitate the comparison with manometric experiments.

The reactions probably proceed according to the following equations:

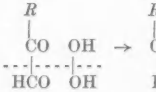


$$CH_3{-}C(=O){-}CH(O.CO.CH_3){-}COOC_2H_5 + H_2O = CH_3{-}C(OH){=}C(OH){-}COOC_2H_5 + CH_3COOH, \quad \text{.....(1)}$$

$$CH_3{-}C(OH){=}C(OH){-}COOC_2H_5 + O_2 = CH_3{-}C(=O){-}C(=O){-}COOC_2H_5 + H_2O_2, \quad \text{.....(2)}$$

$$CH_3{-}C(=O){-}C(=O){-}COOC_2H_5 + H_2O_2 = CH_3COOH + HOOC{-}COOC_2H_5. \quad \text{.....(3)}$$

$H_2O_2$ could not be detected in the solution at the end of the reaction. But its intermediate formation in autoxidations is now generally assumed. The suggested action of $H_2O_2$ on ethyl diketobutyrate (3) is supported by the observation of Friedemann [1927] that glyoxals are oxidized by $H_2O_2$ in alkaline solution according to the general scheme:

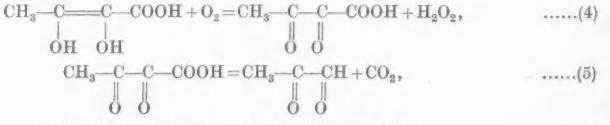


$$\begin{matrix} R \\ | \\ CO \\ | \\ HCO \end{matrix} \quad \begin{matrix} OH \\ | \\ OH \end{matrix} \rightarrow \begin{matrix} R \\ | \\ COOH. \\ + \\ HCOOH \end{matrix}$$

(2) *Autoxidation of hydroxyacetoacetic acid.* In marked contrast to ethyl hydroxyacetoacetate the autoxidation of the acid is slow in $NaHCO_3$. In $N/6$ NaOH, however, 1 mol. $O_2$ is rapidly taken up and 1 mol. $CO_2$ is formed. No $H_2O_2$ is present in the solution at the end of the reaction.

0·3 ml. $M/20$ hydroxyacetoacetate which with aniline yielded 325 $\mu$l. $CO_2$ was tipped into 2 ml. $N/6$ NaOH and absorbed 312 $\mu$l. $O_2$ in 60 min. On acidification with 0·3 ml. 10% acetic acid ($p$H = 5) 310 $\mu$l. $CO_2$ were evolved (corrected for blank).

No $CO_2$ was formed from a solution treated in exactly the same way in an atmosphere of pure $N_2$. The decarboxylation must therefore occur after oxidation. The process might be formulated thus:



$$CH_3{-}C(OH){=}C(OH){-}COOH + O_2 = CH_3{-}C(=O){-}C(=O){-}COOH + H_2O_2, \quad \text{.....(4)}$$

$$CH_3{-}C(=O){-}C(=O){-}COOH = CH_3{-}C(=O){-}CH + CO_2, \quad \text{.....(5)}$$

$$CH_3{-}CO{-}CHO + H_2O_2 = CH_3COOH + HCOOH. \quad \text{.....(6)}$$

According to this scheme the consumption of 1 mol. alkali (after boiling out the $CO_2$) and the formation of 1 mol. acetic acid and 1 mol. formic acid would be expected. In fact however no alkali has been bound at the end of autoxidation. Further analysis, carried out under mild conditions (distillation *in vacuo*), shows that only 1 mol. volatile acid has been formed and this consists entirely of formic acid. But if the solution is heated with acid for several hours and then analysed for volatile acids, 2 mol. are found of which one is formic acid. Obviously a neutral polymerization product is first formed which quantitatively yields acetic acid on heating with acid. Its nature is discussed below.

2 ml. $M/20$ hydroxyacetate (= 2580 $\mu$l. by the aniline method) and 0·3 ml. 10% NaOH were mixed and immediately titrated with $N/10$ HCl at 100° against methyl red: 7·5 ml. were used. An identical sample was titrated after 5 hr. incubation at 25°, when 2620 $\mu$l. $O_2$ had been absorbed. Again 7·5 ml. $N/10$ HCl were used.

A third sample of 2 ml. $M/20$ hydroxyacetoacetate + 0·3 ml. 10% NaOH was, after exhaustive autoxidation, analysed for volatile acids as follows: the solution was acidified with $H_3PO_4$ and

made up to about 30 ml. It was now distilled *in vacuo* until the residue was concentrated to about 5 ml.; 10 ml. water were added and the solution again concentrated to 5 ml. This process was repeated five times. A further distillate was free from acid. The combined distillates were titrated with $N/10$ $Ba(OH)_2$. Found: before autoxidation: 2600 $\mu$l. volatile acid; after autoxidation: 5220 $\mu$l. volatile acid. Therefore 1 mol. volatile acid had been formed during autoxidation.

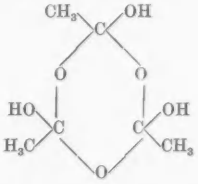
The neutralized distillates were concentrated on the water bath to about 6 ml. After cooling they were acidified with $N$ $H_2SO_4$, made up to 10 ml. with a solution of 10% $MnSO_4$ in 10$N$ $H_2SO_4$ and filtered. An aliquot of 2 ml. was taken from the filtrate and transferred to the main part of a manometer vessel, the side bulb of which contained 0·5 ml. 1·5$N$ $KMnO_4$. On tipping, formic acid was oxidized, liberating 1 mol. $CO_2$. The reaction was completed after 3 hr. at 25°. Formic acid found before autoxidation: 0; after autoxidation: 2540 $\mu$l.

1 ml. $M/10$ hydroxyacetoacetate (=2100 $\mu$l. by the aniline method) +7 ml. $N/10$ NaOH were left in an open flask at room temperature for 24 hr. The solution was neutralized and made up to 12 ml. 6 ml. were analysed for volatile acids immediately, the remaining 6 ml. after heating on the water bath with 0·6 ml. 10$N$ $H_2SO_4$.

| Exp. | 1 | 2 | |
|---|---|---|---|
| Unheated | 4200 $\mu$l. | 4230 $\mu$l. | Volatile acid. |
| Heated 1 hr. | 5250 $\mu$l. | Heated 4 hr. 6440 $\mu$l. | |

No oxalic acid was found after autoxidation of hydroxyacetoacetic acid.

*Polymerization.* It is obvious that the failure to detect acetic acid by mild analysis and its gradual appearance after heating with acid, as well as the formation of only 1 mol. acid instead of two, are due to polymerization of hydroxyacetoacetic acid or of one of its oxidation products in alkaline medium. The constitution of the product of condensation as it appears after complete autoxidation has not been further investigated, but in view of the fact that it is built up of the $CH_3$—CO— moiety of the molecule only, that it is not acid and that it yields acetic acid quantitatively after heating with acid, the following formula of a trihydroxyparaldehyde may be tentatively suggested.

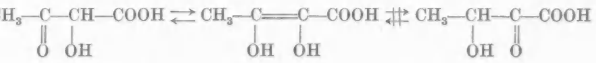


A very similar formula has been proposed by Evans & Waring [1926] for the trimeric form of hydroxypyruvic aldehyde and the paraldehyde type of condensation is also favoured by Levene & Walti [1931] for crystalline dimeric lactaldehyde. In aqueous alkaline solution lactaldehyde, according to Dworzak & Prodinger [1928], polymerizes to a trimolecular product. Hydroxypyruvic and lactic aldehydes are, like hydroxyacetoacetic acid, ketohydroxy-compounds.

*The ketohydroxy-enediol tautomerism*

(1) *The equilibrium.* The concentration of enediol form in a freshly prepared $N/10$ solution of hydroxyacetoacetic acid at $p$H 6·5 and 0° has been determined by iodine titration and found to be 1·35%. It is thus considerably higher than the enol content of a solution of acetoacetic acid under similar conditions, which was found by Grossmann [1924] to be under 0·1%. The concentration of the enediol form is of course greatly increased in a more alkaline medium and, if

autoxidation has been prevented, the ketohydroxy form will be reformed on neutralization. If an equilibrium exists between the three tautomerides, one would expect the enediol form to be converted partly into the α-keto-β-hydroxy- and partly to the α-hydroxy-β-keto-compound. α-Keto-β-hydroxybutyric acid has not yet been described, but it is reasonable to assume that, as an α-ketonic acid, it will not be decarboxylated by aniline, whereas it may be attacked by carboxylase. A solution of hydroxyacetoacetate was therefore incubated in $N/10$ NaOH at 25° for 2 hr. in an atmosphere of pure $N_2$ and subsequently neutralized. Aliquots were then subjected to the action of aniline or carboxylase. It was found that the $CO_2$ evolution on addition of aniline had not decreased substantially, whilst carboxylase had no effect. A small increase of $CO_2$ output was due to non-enzymic catalysis of decarboxylation of the β-ketonic acid, since it occurred also with a boiled sample of carboxylase. It appears therefore that of the tautomeric equilibria:



only the left-hand half exists in aqueous solution.

A similar case is that of ascorbic acid; 2-ketogulonic acid does not form a true equilibrium with the enediol (ascorbic acid) and requires very drastic treatment for conversion into the latter. Ascorbic acid, on the other hand, forms a true equilibrium with 3-ketogulonic acid, which lies almost entirely on the side of the former [Reichstein & Grüssner, 1934].

(2) *The velocity of oxidation.* If iodine is added to a solution of hydroxyacetoacetic acid the first few drops are reduced very quickly, until all the preformed enediol component has been oxidized. A further drop is reduced only very slowly. The same observation is made if the titration is carried out with an indicator, e.g. 2:6-dichlorophenolindophenol. It follows that, in certain cases at least, the reaction of the enediol form with the oxidant is fast compared with the velocity of enolization. Therefore the velocity of oxidation is a measure of the ease with which the compound is enolized. It was of interest to compare the tendencies to enolization of ethyl hydroxyacetoacetate, hydroxyacetoacetate and acetol by measuring the velocities of oxidation under identical conditions. Quite generally the following order was found: ethyl hydroxyacetoacetate $\gg$ hydroxyacetoacetate $\gg$ acetol.

(*a*) The autoxidation of ethyl hydroxyacetoacetate in $N/10$ $NaHCO_3$ is complete in less than 2 hr. (Fig. 4). In other experiments it was even considerably faster. The autoxidation of hydroxyacetoacetate on the other hand is very slow in neutral or weakly alkaline solution. It varies somewhat from one experiment to another, probably owing to the amount of heavy metals accidentally present.

In Fig. 5 the autoxidations of hydroxyacetoacetate and of acetol in $N/10$ NaOH are compared; whereas the autoxidation of hydroxyacetoacetate is complete after 15 min. that of acetol has only reached about 60 % after $3\frac{1}{2}$ hr. The solutions of acetol used in these experiments were prepared by boiling a solution of hydroxyacetoacetate for 5 min. under reflux. Equal quantities of the unboiled and boiled solution were taken for comparison.

(*b*) If carried out in a bicarbonate-$CO_2$ buffer many oxidation-reduction reactions can be followed manometrically by the liberation of acid. Thus the reduction of 1 mol. methylene blue is accompanied by the liberation of 1 mol. $CO_2$ from the $NaHCO_3$ [Reid, 1931]. Preliminary experiments with Thunberg

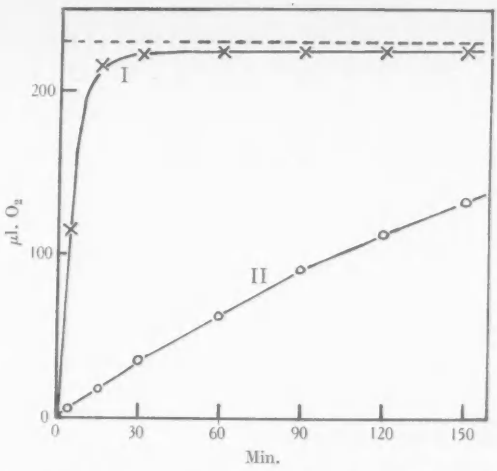



Fig. 5. Autoxidation of hydroxyacetoacetic acid (I) and acetol (II) in $N/10$ NaOH. The broken line indicates the quantity of $CO_2$ evolved from the same volume of the hydroxyacetoacetic acid solution after addition of aniline.

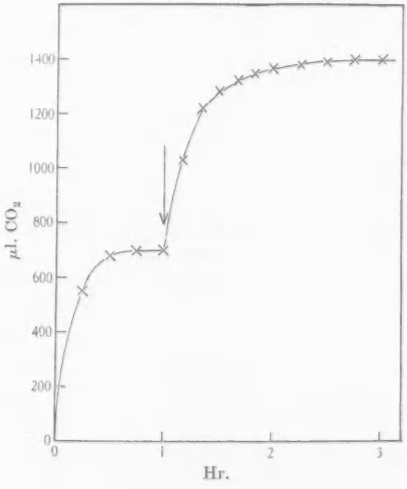



Fig. 6.

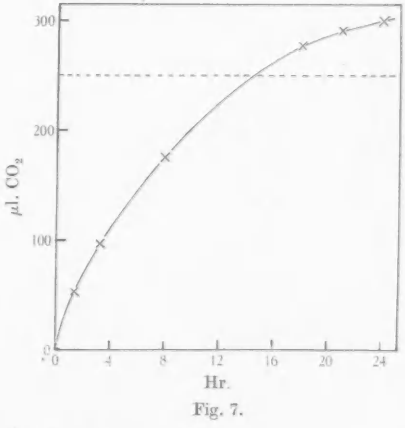



Fig. 7.

Fig. 6. Oxidation of ethyl hydroxyacetoacetate by methylene blue.

Warburg manometer filled with Clerici solution was used. The manometer vessel contained 3 ml. $M/40$ $NaHCO_3$ in the main part, 0·004 ml. of ethyl acetoxyacetoacetate in side bulb I and 0·5 ml. 3% methylene blue in side bulb II. The gas space contained purified $N_2$ with 5% $CO_2$. The ester was tipped in at the beginning and the methylene blue after 1 hr. (arrow). 25°.

Fig. 7. Oxidation of hydroxyacetoacetic acid by methylene blue.

The main part of the manometer vessel contained 2 ml. $M/40$ $NaHCO_3$ and 1 ml. 1·2% methylene blue. The gas space was filled with purified $N_2$/5% $CO_2$. 0·1 ml. $M/10$ hydroxyacetoacetate was tipped from the side bulb at the beginning of the experiment which was carried out at 25°. The broken line indicates 1 mol. as determined by the aniline method.

tubes showed that the following indicators were completely reduced by an excess of hydroxyacetoacetate: phenolindophenol, 2:6-dichlorophenolindophenol, thionine, brilliant cresyl blue, methylene blue, pyocyanine, methyl Capri blue, indigotrisulphonate, though in most cases the process is very slow and takes many hours. Phenosafranine and neutral red were not reduced. Figs. 6 and 7 show the reduction of methylene blue by ethyl hydroxyacetoacetate and by hydroxyacetoacetate in manometric experiments ($M/40$ bicarbonate-$CO_2$ buffer $p$H 7·4, 25°). In Fig. 6 the hydrolysis of ethyl acetoxyacetoacetate is recorded in the first period; the acid liberated indicates the exact amount of ethyl hydroxyacetoacetate present. When the hydrolysis is finished, the methylene blue is tipped in from a side bulb. 1 mol. methylene blue is reduced in about 2 hr. With hydroxyacetoacetate the reaction is much slower; 1 mol. acid is formed after 15 hr., but the reaction still continues. It has not been possible to observe the end value, but it is probable that it would have reached 2 mol., especially if it is remembered that the spontaneous decomposition of hydroxyacetoacetate is appreciable over such long periods. No reduction of methylene blue was observed with acetol under the same conditions.

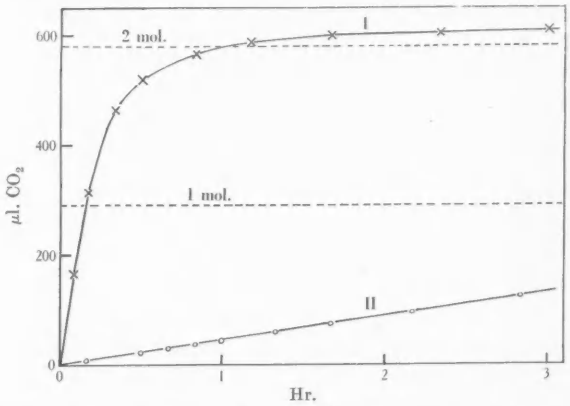



Fig. 8. Oxidation of hydroxyacetoacetic acid by a copper-bicarbonate solution (I) and by a ferricyanide bicarbonate solution (II).

The oxidation of hydroxyacetoacetate by a copper-bicarbonate or a ferricyanide-bicarbonate solution can also be followed manometrically (Fig. 8). In the case of the copper reduction the reaction can be schematically written thus:

$$2\,Cu^{++} + —CO—CHOH— \;=\; 2\,Cu^{+} + —CO—CO— + 2\,H^{+}.$$

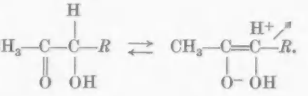
2 mol. $CO_2$ are actually liberated in the experiment. The reaction is rapid, being complete after 2 hr. The oxidation with ferricyanide is much slower. The underlying reaction is similar [cf. Haas, 1937]:

$$2Fe(CN)_6^{---} + —CO—CHOH— \;=\; 2Fe(CN)_6^{----} + —CO—CO— + 2\,H^{+}.$$

The greater velocity of the reaction with the copper solution than of that with the ferricyanide solution may be explained by the fact that the positive copper ions have a higher affinity to the anion of the acid than the negative ferricyanide ions. Neither the copper nor the ferricyanide solution reacted with acetol under the conditions of the experiment.

The experiments were carried out at 25° as follows: 0·1 ml. of *ca.* $M/10$ hydroxyacetoacetate (the exact concentration of which was previously determined by aniline analysis) and 2 ml. $M/10$ $NaHCO_3$ were placed in the main part of the manometer vessel. The gas space was filled with 5% $CO_2$ in $N_2$, purified over hot copper. After equilibration 0·4 ml. of the copper or ferricyanide solution was tipped from the side bulb. The copper solution had the following composition: 4 g. copper acetate, 15 g. Rochelle salt, 3·2 g. $NaHCO_3$ made up to 100 ml. The ferricyanide solution contained 5 g. $K_3Fe(CN)_6$ dissolved in 100 ml. $M/10$ $NaHCO_3$.

The fact that the tendency to enolization increases from acetol to hydroxyacetoacetic acid and from hydroxyacetoacetic acid to ethyl hydroxyacetoacetate is easily understood in the light of the electronic theory. Enolization is actually a dissociation according to the scheme:



If $R$ is a carboxyl or a carbethoxyl group the mobility of the dissociable proton will be greater than if $R$ is a H-atom. On the other hand the negative charge at the carboxylate group has a restraining influence on the release of the proton, compared with the electrically neutral carbethoxyl group. The same reasons explain why if the enediol acid is converted into the ketohydroxy-acid the α-hydroxy-β-keto-acid only is formed. The tendency to release an electron will be stronger at the α- than at the β-position and the free proton will be oriented towards the negative forces of the carboxylate group.

*The estimation of acetol by reduction methods*

For tissue experiments a method was required by which the sum of hydroxyacetoacetic acid and the acetol formed by decarboxylation could be estimated. This is possible by using any of the current methods for the estimation of glucose by reduction. If a standard solution of acetol, prepared as described below, is heated for 15 min. in a boiling water bath with the copper solution of Shaffer-Hartmann [Peters & Van Slyke, 1932, p. 466, reagent 1], 2 $Cu^{++}$ are reduced for 1 mol. acetol, corresponding to $\frac{1}{2}$ mol. $O_2$. 1 ml. $N/100$ thiosulphate therefore indicates 112 μl. acetol in the final titration.

If a standard solution of acetol is heated for 30 min. in a boiling water bath with 0·05 $N$ $K_3Fe(CN)_6$ in 7% $Na_2CO_3$, acidified after cooling and titrated with $N/100$ ceric sulphate, using setopaline as indicator [Miller & Van Slyke, 1936], 4 mol. ferricyanide are reduced by 1 mol. acetol, corresponding to 1 mol. $O_2$. 1 ml. $N/100$ ceric sulphate therefore indicates 56 μl. acetol.

Standard solution of acetol ($M/50$): 520 mg. recrystallized bis-acetoldimethylether [Nef, 1904], M.P. 127°, were heated under reflux with 25 ml. $N$ HCl on the water bath for 30 min., cooled, neutralized and made up to 250 ml.

1 ml. $M/50$ acetol + 3 ml. Shaffer-Hartmann solution used 2·60 ml. $N/100$ thiosulphate. 3 ml. Shaffer-Hartmann solution used 6·60 ml. Difference: 4·00 ml. $N/100$ thiosulphate.

1 ml. $M/50$ acetol + 3 ml. ferricyanide solution, titrated with $N/100$ ceric sulphate, used 8·00 ml.

If a freshly prepared solution of hydroxyacetoacetate is converted into acetol and $CO_2$ by boiling, the ferricyanide method gives values which agree closely with the amount of $CO_2$ liberated by aniline from the unboiled solution. The values obtained by the copper method are, however, low (70–80%) for reasons which are not clear. This is especially surprising since the oxidation by

copper gave a theoretical result in the manometric experiments referred to above.

0·2 ml. of *ca.* *M*/10 hydroxyacetoacetate yielded 552 $\mu$l. $CO_2$ on addition of aniline.

0·2 ml. of this solution, after boiling + 3 ml. Shaffer-Hartmann solution, 15 min. at 100°: titrated 3·45 ml. *N*/100 thiosulphate = 386 $\mu$l. (70 %).

0·2 ml. of this solution + 3 ml. ferricyanide solution, 30 min. at 100°: titrated 9·45 ml. *N*/100 ceric sulphate = 530 $\mu$l. (96 %).

This estimation is of course not very specific. Separation from other reducing substances, especially sugars, was not possible. A blank with an equal amount of tissue but without hydroxyacetoacetate was run in every experiment and the result corrected accordingly. This blank is small with most sliced tissues, except liver.

The ferricyanide method cannot be used in presence of acetoacetic acid or acetone, since acetone reacts with alkaline ferricyanide at 100°.

*Conversion of acetol into lactic acid*

Denis [1907] and Evans & Hoover [1922] have shown that acetol is oxidized by $KMnO_4$ at room temperature in neutral or weakly acid solution yielding equimolecular amounts of acetic acid and $CO_2$. The reaction can be studied manometrically, but it is so slow that it is not convenient for analytical purposes. No volatile carbonyl compounds are formed in the oxidation of acetol by $KMnO_4$; acetol does not therefore interfere with lactic acid estimation, nor does hydroxyacetoacetic acid. If, however, a solution of acetol is subjected to the copper-lime treatment, it is converted almost quantitatively into lactic acid, being first oxidized to methylglyoxal [cf. Denis, 1907] which, in alkaline solution, is converted into lactic acid by intramolecular dismutation. It is important to realize this if the formation of lactic acid is studied in presence of acetol. A freshly prepared solution of hydroxyacetoacetic acid, free from acetol, does not form any lactic acid when treated with copper-lime. The acid is apparently completely removed from the solution by the copper-lime treatment.

10 ml. *M*/300 acetol, containing 790 $\mu$l., analysed for lactic acid by the method of Friedemann & Kendall [1929]: no lactic acid.

The same + 1 ml. 25 % $CuSO_4$ + 1 ml. 12·5 % $Ca(OH)_2$. Left at room temperature for 30 min. with repeated shaking. Filtered and analysed for lactic acid: 635 $\mu$l. lactic acid (80 %).

## II. METABOLISM IN ANIMAL TISSUES

Clutterbuck & Raper [1926] suggested that the physiological oxidation of acetoacetic acid follows the same path as the oxidation *in vitro* by $H_2O_2$ in alkaline solution. According to this scheme acetoacetic acid would be oxidized to hydroxyacetoacetic acid and further to diketobutyric acid. This substance would be decarboxylated to methylglyoxal which would be converted into lactic acid. Some support was given to this scheme by experiments of Haarmann [1935] who reported formation of lactic acid from $\beta$-hydroxybutyric acid by various tissues under anaerobic conditions.

Since the decarboxylation of diketobutyric acid can be assumed to take place spontaneously and the conversion of methylglyoxal into lactic acid is a

well-known enzymic reaction, the steps to be proved are (1) the oxidation of acetoacetic acid to hydroxyacetoacetic acid, (2) the oxidation of hydroxyacetoacetic acid to diketobutyric acid or methylglyoxal. The preparation of solutions of hydroxyacetoacetate, described in the preceding section, made it possible to test the second of these reactions with tissues. If hydroxyacetoacetic acid is oxidized by tissues, increased respiration and aerobic disappearance of the substrate are to be expected. It is known that the tissue where the oxidative breakdown of acetoacetic acid is highest is kidney. This is true for the intact perfused organ [Snapper & Grünbaum, 1927] as well as for kidney slices [Quastel & Wheatley, 1935; Edson & Leloir, 1936]. Special attention has therefore been directed to experiments with kidney.

*Methods.* Tissue experiments were carried out at 37·5° with the Warburg apparatus. The results are expressed in the conventional $Q$-units.

*Respiration of rat tissues in presence of hydroxyacetoacetic acid*

When measuring the respiration of tissue slices in presence of hydroxyacetoacetic acid it is necessary to correct the results for the small autoxidation of the latter. This has been done with the results collected in Table II. It is also

Table II. *Respiration of rat tissues*

Phosphate-saline. $O_2$. HAA = Hydroxyacetoacetic acid

| Exp. | Tissue | Substrate | $Q_{O_2}$ 1st hr. | 2nd hr. | 3rd hr. |
|---|---|---|---|---|---|
| 1 | Kidney (slices) | 0 | −15·3 | −10·5 | − 9·3 |
| | | *M*/100 HAA | −21·7 | −16·7 | −15·5 |
| | | *M*/100 acetic acid | −21·5 | −16·7 | −16·8 |
| 2 | | 0 | −19·4 | −15·6 | −12·3 |
| | | *M*/100 HAA | −28·6 | −23·5 | −13·7 |
| | | *M*/100 acetic acid | −25·6 | −23·6 | −22·3 |
| 3 | Brain (slices) | 0 | − 6·5 | − 3·3 | − 1·2 |
| | | *M*/100 HAA | − 6·0 | − 2·2 | − 1·1 |
| 4 | Liver (slices) | 0 | − 9·2 | − 8·7 | |
| | | *M*/100 HAA | −13·7 | −13·1 | |
| 5 | | 0 | − 9·5 | − 8·0 | |
| | | *M*/100 HAA | −15·3 | −14·5 | |
| | | *M*/100 acetic acid | −13·9 | −12·6 | |
| 6 | Heart (slices) | 0 | − 3·4 | − 2·6 | |
| | | *M*/100 HAA | −10·5 | −10·6 | |
| 7 | | 0 | − 3·1 | − 2·8 | |
| | | *M*/100 HAA | − 8·5 | − 6·4 | |
| | | *M*/100 acetic acid | − 6·7 | − 7·1 | |
| 8 | Testis | 0 | − 6·8 | − 4·9 | |
| | | *M*/100 HAA | − 8·2 | − 3·7 | |
| 9 | Jensen rat sarcoma (slices) | 0 | −11·5 | − 9·7 | |
| | | *M*/150 HAA | −11·1 | − 8·2 | |
| | | *M*/150 acetic acid | − 9·5 | − 8·4 | |

to be kept in mind that the solutions of hydroxyacetoacetate contain an equimolecular amount of acetate. An increase of respiration may be due to oxidation of either substrate. Only if the respiration in presence of hydroxyacetoacetic acid were considerably higher than in presence of an equimolecular amount of acetic acid could the effect be attributed with certainty to hydroxyacetoacetic acid. But the increase of respiration where it is observed is in most cases not or only slightly higher than with an equimolecular quantity of acetic acid.

*Disappearance of hydroxyacetoacetic acid added to rat tissues*

Hydroxyacetoacetic acid disappears very quickly if incubated with slices of rat kidney. The rate of disappearance is about the same in presence as in absence of $O_2$ (Table III).

Table III. *Disappearance of hydroxyacetoacetic acid in presence of slices of rat kidney*

1 hr. incubation in bicarbonate-saline. Amount of hydroxyacetoacetic acid present at start: 550 $\mu$l. Estimation of hydroxyacetoacetic acid present at the end with aniline at 25°

| Mg. dry wt. | Gas phase | $\mu$l. HAA final | Disappearance due to tissue $\mu$l. HAA | $Q_{HAA}$ |
|---|---|---|---|---|
| 0 | $O_2$/5% $CO_2$ | 470 | — | — |
| 5·61 | $O_2$/5% $CO_2$ | 226 | 244 | −43·5 |
| 6·64 | $N_2$/5% $CO_2$ | 152 | 318 | −47·7 |

These results were obtained with the aniline method which measures the disappearance of the $\beta$-ketonic acid only. This disappearance might be due to simple decarboxylation: in this case corresponding amounts of acetol and bicarbonate would be found. Or the $\beta$-ketonic acid might have been converted into another compound which is not decarboxylated by aniline, e.g. an intramolecular dismutation to $\alpha$-keto-$\beta$-hydroxybutyric acid or a reduction to dihydroxybutyric acid. This possibility can be excluded, since the amount of bicarbonate formed aerobically as well as anaerobically accounts for the disappearance of $\beta$-ketonic acid. The sum of $\beta$-ketonic acid and bicarbonate remains constant.

The experiments were done with four vessels provided with two side bulbs. Unbuffered, bicarbonate-poor Krebs-Ringer solution was used as suspension fluid. The gas space was filled with $O_2$ or $N_2$. Vessel I contained neither substrate nor tissue, vessel II contained hydroxyacetoacetic acid without tissue, vessel III contained tissue without substrate and vessel IV tissue + hydroxyacetoacetic acid. After 1 hr. incubation at 37·5° the tissue was removed and 0·3 ml. 3 *M* acetate buffer *p*H 4·6 was placed in one side bulb and 0·4 ml. aniline solution in the other side bulb. The manometers were now shaken in a bath at 25° and bicarbonate and $\beta$-ketonic acid contents were subsequently analysed by tipping in first the acetate buffer and then the aniline solution (see Table IV).

Table IV. *Formation of bicarbonate and disappearance of hydroxyacetoacetic acid in presence of slices of rat kidney*

| Mg. dry wt. | Gas phase | $\mu$l. bicarbonate formed | $\mu$l. HAA final | Sum |
|---|---|---|---|---|
| 0 | $O_2$ | 58 | 206 | 264 |
| 5·38 | $O_2$ | 164·5 | 103 | 267·5 |
| 0 | $N_2$ | 42·5 | 218 | 260·5 |
| 5·94 | $N_2$ | 200 | 48 | 248 |

It follows that the disappearance of hydroxyacetoacetic acid is accompanied by decarboxylation. At least as far as the aerobic experiments are concerned, however, this decarboxylation could take place after oxidation of hydroxyacetoacetic acid to diketobutyric acid or, alternatively, acetol could be oxidized after the decarboxylation. In both these cases one would find a decrease of the sum of hydroxyacetoacetic acid + acetol (= "total acetol").

The disappearance of total acetol has been examined in a series of experiments using both reduction methods described (Table V).

At the end of the experiment 0·2 ml. 2*N* HCl was added. The tissue was removed and the solution neutralized with *N* NaOH. The solution was made up to about 8 ml. and heated in a boiling water bath for 5 min., after a little kieselguhr had been added. The filtered solution was analysed for acetol. Blanks without substrate were carried out in every case and the results corrected in proportion to the dry weights of tissue.

Table V. *Disappearance of total acetol in rat tissues*

2 hr. incubation in $O_2$ if not stated otherwise

| Tissue | mg. dry wt. | μl. total acetol final | Decrease μl. | Remarks |
|---|---|---|---|---|
| Exp. 1. Total acetol present at start, 443 μl. Ferricyanide method | | | | |
| 0 | — | 372 | 71 | — |
| Brain | 14·94 | 388 | 55 | — |
| Kidney | 10·85 | 400 | 43 | — |
| Liver | 18·87 | 295 | 148 | — |
| Heart | 17·02 | 367 | 76 | — |
| Testis | 49·41 | 440 | 3 | — |
| Exp. 2. Total acetol present at start, 390 μl. Ferricyanide method | | | | |
| 0 | — | 375 | 15 | — |
| Liver | 15·69 | 300 | 90 | — |
| Heart | 14·90 | 325 | 65 | — |
| Exp. 3. Total acetol present at start, 440 μl. Copper method | | | | |
| 0 | — | 413 | 27 | — |
| Kidney | 9·49 | 438 | 2 | — |
| Testis | 37·57 | 432·5 | 7·5 | — |
| Heart | 26·57 | 360 | 80 | — |
| Exp. 4. Total acetol present at start, 475 μl. Ferricyanide method | | | | |
| 0 | — | 377 | 98 | — |
| 0 | — | 472 | 3 | Anaerobic exp. |
| Liver | 16·16 | 440 | 35 | — |
| | 16·13 | 475 | 0 | Boiled soln. of HAA |
| | 15·75 | 472 | 3 | Anaerobic exp. |
| Heart | 30·23 | 385 | 90 | — |
| | 24·85 | 475 | 0 | Boiled soln. of HAA |
| | 31·23 | 466 | 9 | Anaerobic exp. |
| Exp. 5. Total acetol present at start, 368 μl. Ferricyanide method | | | | |
| Jensen rat | 0 | 344 | 24 | — |
| sarcoma | 11·61 | 350 | 18 | — |

The interpretation of these results which are typical of a greater number of experiments is complicated by the fact that there is sometimes considerable disappearance of total acetol even in absence of tissue, probably owing to autoxidation of hydroxyacetoacetic acid. In most cases this disappearance is not increased but rather reduced by the presence of tissues. It is impossible to say how much of the disappearance found in presence of tissues is due to enzymic processes and how much to autoxidation. Liver and heart are the only tissues with which the disappearance is sometimes definitely increased, but even this is not a constant observation. With kidney the result was always clearly negative; yet kidney is the tissue where the oxidative removal of hydroxyacetoacetic acid should be highest if this substance is an intermediary of acetoacetic acid metabolism.

Summarizing the evidence so far presented it appears that there is no effect on respiration which could with certainty be attributed to hydroxyacetoacetic acid and that a possible small oxidative disappearance is observed only with liver and heart. The only effect which is observed in presence of kidney is a rapid

decarboxylation to acetol and bicarbonate. It is of course possible that this decarboxylation is too rapid to allow the hydroxyacetoacetic acid added to the suspension fluid to penetrate to the centre of oxidation within the cell.

*Formation of hydroxyacetoacetic acid by tissues*

A series of experiments was undertaken to test the first step in the hypothetical sequence of reactions which lead from acetoacetic acid to lactic acid, viz. the oxidation of acetoacetic acid to hydroxyacetoacetic acid. It is quite clear from earlier work [Snapper & Grünbaum, 1927; Friedemann, 1936; Quastel & Wheatley, 1935; Edson & Leloir, 1936] that the body is able to oxidize acetoacetic acid and that this process is specially active in kidney, but we are still quite ignorant about the intermediary stages of the process.

The anaerobic disappearance of acetoacetic acid was first investigated, together with the formation of $\beta$-hydroxybutyric acid. Edson & Leloir presented figures for the anaerobic disappearance of acetoacetic acid in various tissues and under varying conditions, but they have not examined the simultaneous formation of $\beta$-hydroxybutyric acid. Quastel & Wheatley on the other hand published two experiments with guinea-pig kidney where the respiration was poisoned with HCN. They found in one experiment a formation of $\beta$-hydroxybutyric acid which accounted for 78 % of the acetoacetic acid disappearing, but in the other experiment it accounted only for 52 %.[1] I have found quite regularly that the $\beta$-hydroxybutyric acid formed accounted only for about 50 % of the acetoacetic acid disappearing (Table VI).

The estimation of acetoacetic acid and $\beta$-hydroxybutyric acid was carried out in the same solution by the method previously described [Weil-Malherbe, 1937]. A yield of 76–78 % of acetone from $\beta$-hydroxybutyric acid was obtained with great regularity if the following details were adhered to: to the solution in the distilling flask were added 6 ml. 66 vol. % $H_2SO_4$ and water to make a total volume of 30 ml. The solution was now heated to boiling and 10 ml. 0·1 % $K_2Cr_2O_7$ were slowly added from the dropping funnel. The flame was reduced so that the solution was just kept boiling and the rate of distillation was not higher than 1 drop in 4 sec. The distillation of the 25 ml. should be finished in not less than 30 min. Water is added from the dropping funnel to keep the volume in the flask constant.

Table VI. *Dismutation of acetoacetic acid with rat tissues*

Bicarbonate saline. $N_2/5\%$ $CO_2$. $M/100$ acetoacetate

| Tissue | $Q_{\text{Acetone}}$ | $Q_{\beta\text{-Hydroxybut.}}$ |
|---|---|---|
| Brain | −0·67 | 0·33 |
| | −0·55 | 0·30 |
| Testis | −0·98 | 0·48 |
| | −0·69 | 0·35 |
| Kidney | −1·57 | 0·81 |
| | −1·64 | 0·85 |
| + $M/100$ $l(+)$alanine | −2·30 | 1·24 |

It is reasonable to assume that, if 50 % of the acetoacetic acid disappearing is reduced, the other 50 % is oxidized, i.e. that the anaerobic disappearance of acetoacetic acid is due to a dismutation, as is the case with other keto-acids [cf. Weil-Malherbe, 1937].

Since acetoacetic acid disappears only in proportion to the velocity with which it can be reduced, its removal is primarily an oxidative process. This is a

[1] Quastel & Wheatley's experiments in presence of glucose and pyruvate are deliberately left out of the discussion; here the reactions are complicated by a possible dismutation between pyruvic and acetoacetic acids and also by a possible formation of $\beta$-hydroxybutyric acid from pyruvic acid [Krebs & Johnson, 1937].

valuable clue since it excludes other non-oxidative reactions which have been suggested, such as hydrolytic cleavage into two molecules of acetic acid or hydration to dihydroxybutyric acid [Haarmann & Schroeder, 1938].

The anaerobic $CO_2$ production of tissue incubated anaerobically in bicarbonate-saline is not at all or only insignificantly greater in presence than in absence of acetoacetic acid. The dismutation of acetoacetic acid is therefore not accompanied by decarboxylation or acid formation.

All these facts could be explained if it were possible to identify the oxidative equivalent of the dismutation with hydroxyacetoacetic acid and it was these experiments which originally led to the present investigation. But all efforts to detect the formation of hydroxyacetoacetic acid from acetoacetic acid failed. If hydroxyacetoacetic acid were really formed from acetoacetic acid, its own oxidation should be sufficiently checked under anaerobic conditions to allow some accumulation. The measurement of the rate of dismutation allowed an estimate of the quantity of oxidative equivalent that could be expected and the dimensions of the experiment were so chosen that at least 100 $\mu$l. of the oxidative equivalent were formed (about 100 mg. dry wt. of slices in a 2 hr. experiment). At the end of the experiment all enzymic processes were stopped by acidification before admission of $O_2$. After removal of the tissue the solution was deproteinized by boiling with kieselguhr and filtered. The filtrate was then analysed by one of the following methods: (1) heating with Shaffer-Hartmann's copper solution did not reveal the presence of a reducing substance; (2) the filtrate was acidified and distilled and the distillate was tested with *o*-aminobenzaldehyde for acetol; no acetol could ever be detected, though the test was intensely positive if model estimations with the expected amount of acetol were performed; even a few $\mu$l. acetol produced obvious blue fluorescence in the light of a mercury lamp; (3) the filtrate was analysed for lactic acid by the method of Friedemann & Kendall [1929]; if hydroxyacetoacetic acid had been further oxidized even under anaerobic conditions, perhaps by undergoing a dismutation itself, the product formed would be lactic acid; no formation of lactic acid was, however, observed (Table VII). The analysis was sometimes carried out with the kieselguhr filtrate without further treatment, in other experiments after treatment with copper-lime. In the latter case any acetol present would have been partly estimated as lactic acid.

If hydroxyacetoacetic acid were formed from acetoacetic acid in the body, one would expect that just as in the case of acetoacetic acid it would partly escape further oxidation by spontaneous decarboxylation. Acetol should therefore appear in the urine of persons with ketosis where the body has to deal with large amounts of acetoacetic acid. Through the courtesy of Dr F. K. Herbert I examined the urine of a patient in diabetic coma. The freshly passed urine

Table VII. *Formation of lactic acid by slices of rat kidney in presence of acetoacetic and hydroxyacetoacetic acids*

Bicarbonate-saline. 2 hr. HAA = hydroxyacetoacetic acid

| Substrate | Gas phase | Pre-treatment | $Q_{Lact.}$ |
|---|---|---|---|
| 0 | $O_2$/5% $CO_2$ | Kieselguhr | 0·31 |
| *M*/50 acetoacetate | — | — | 0·24 |
| *M*/100 HAA | — | — | 0·34 |
| 0 | $N_2$/5% $CO_2$ | Kieselguhr | 1·5 |
| *M*/50 acetoacetate | — | — | 1·4 |
| *M*/100 HAA | — | — | 1·8 |
| 0 | $N_2$/5% $CO_2$ | Copper-lime | 0·59 |
| *M*/50 acetoacetate | — | — | 0·53 |

which had a strongly positive $FeCl_3$ reaction was acidified and distilled, and the distillate was tested with *o*-aminobenzaldehyde; the test was negative.

Under aerobic conditions considerable quantities of a reducing substance are formed from acetoacetic acid. This substance does not give the fluorescence test with *o*-aminobenzaldehyde and is therefore not identical with acetol. Its nature is being further investigated. No formation of lactic acid was detected in aerobic experiments (Table VII).

The failure to detect hydroxyacetoacetic acid or acetol amongst the oxidation products of acetoacetic acid in tissue experiments does not exclude conclusively the possibility of their intermediate formation. It is still possible to argue that nascent hydroxyacetoacetic acid disappears too quickly or that our means of detection are too inadequate to obtain positive evidence of its intermediate formation. This objection can be effectively answered since it is possible to demonstrate a case where hydroxyacetoacetic acid is indeed formed in kidney slices, viz. by oxidation of threo-1:2-dihydroxybutyric acid. There is no reason to assume that hydroxyacetoacetic acid behaves differently whether it is formed from acetoacetic acid or from dihydroxybutyric acid; therefore if it is found in one reaction it should also be detectable in the other reaction, and if not it may be inferred that it is in fact not formed. These results confirm, in our view definitely, the conclusion that hydroxyacetoacetic acid is not an intermediary of the physiological oxidation of acetoacetic acid.

*The oxidation of* dl-*threo-1:2-dihydroxybutyric acid by slices of rat kidney*

The respiration of slices of rat kidney is increased in presence of *dl*-threo-dihydroxybutyric acid, but not of *dl*-erythrodihydroxybutyric acid (Table VIII).

Table VIII. *Respiration of slices of rat kidney*

| | Phosphate-saline. $O_2$ | | | |
|---|---|---|---|---|
| | $Q_{O_2}$ | | | |
| Substrate | Before tipping substrate 10 min. | 1st hr. | 2nd hr. | 3rd hr. |
| 0 | −26·1 | −19·4 | −15·6 | −12·3 |
| M/50 *dl*-threo-1:2-dihydroxybutyric acid | −24·4 | −27·5 | −22·5 | −18·2 |
| *M*/50 *dl*-erythro-1:2-dihydroxybutyric acid | −25·9 | −20·3 | −16·2 | −13·4 |

After aerobic incubation of rat kidney slices in presence of threodihydroxybutyric acid hydroxyacetoacetic acid or acetol can be detected in the solution by the following three reactions: (1) a positive fluorescence test with *o*-aminobenzaldehyde, (2) an evolution of $CO_2$ upon addition of aniline and (3) a reduction of Shaffer-Hartmann's copper solution or of an alkaline ferricyanide solution. All three reactions are negative with pure solutions of threodihydroxybutyric acid and also with solutions in which tissue had been incubated without addition of this substrate.

Owing to the rapid decarboxylation of hydroxyacetoacetic acid by kidney slices the amount of $\beta$-ketonic acid present after a 2 hr. experiment is only about 20 % of the total acetol, as estimated by the reduction method. It is of course uncertain whether the reducing substances formed are entirely "total acetol". If, however, the fluorescence test is carried out with a quantity of acetol corresponding to the amount found by the reduction test and is compared with a test performed on a solution from a tissue experiment, the fluorescences of both solutions are of very similar intensity.

The formation of "total acetol" has been compared with the disappearance of dihydroxybutyric acid. Both analyses were done on aliquots of the same solution.

1:2-Dihydroxybutyric acid was estimated with periodic acid which oxidizes α-glycols specifically [Fleury & Lange, 1932]. The method used was very similar to the procedure described by Fleury & Fatome [1935] for the estimation of glycerol. To the deproteinized, neutralized solution (about 10 ml.) were added 2 ml. $N/10$ periodic acid and 1 ml. $N/10$ HCl and the mixture was left at room temperature for 15 min. 10 ml. saturated $NaHCO_3$, 2 ml. 0·11 $N$ $NaAsO_2$ in 7 % $NaHCO_3$, 2 ml. 10 % KI and 1 ml. starch indicator were then added. After 10 min. the solution was titrated with $N/100$ $I_2$. It is necessary to pipette the periodic acid and the arsenite very accurately. The difference between $N/100$ iodine used in the experimental solution and that used in the blank indicates the quantity of α-glycol present. 1 ml. $N/100$ $I_2$=112 μl. dihydroxybutyric acid. The method gives theoretical results with pure solutions. It was not possible to separate dihydroxybutyric acid and sugars. The value obtained in the blank, corrected for the dry weight of tissue used, was therefore subtracted from the result. Acetol was removed before the estimation by evaporation of the neutral solution to dryness on a water bath.

Table IX. *Disappearance of* dl-*threo-1:2-dihydroxybutyric acid and formation of "total acetol" by slices of rat kidney*

Phosphate-saline. $O_2$. 2 hr. incubation

| Exp. | $Q_{\text{Dihydroxybut.}}$ | $Q_{\text{Acetol.}}$ |
|---|---|---|
| 1 | −4·2 | 2·4 |
| 2 | −3·6 | 1·5 |

Four large Warburg vessels of about 40 ml. volume with side bulb were used for each experiment. They each contained 10 ml. phosphate saline and about 30 mg. of kidney slices (dry wt.). Vessels II and IV contained in addition 0·3 ml. $M/10$ dihydroxybutyric acid in the side bulb, which was added to the tissue at $t_0$. The reaction was interrupted by acidification in vessels I and II at $t_0$, in vessels III and IV after 2 hr.

It appears from Table IX that the acetol formed, as estimated by the reduction method, accounts for about half of the dihydroxybutyric acid disappearing.

Whether this reaction is of physiological significance or whether it is merely due to a limited specificity of the β-hydroxybutyric dehydrogenase or another enzyme will not be discussed. It is important however that hydroxyacetoacetic acid or acetol, when really formed, accumulate to a considerable extent and can be detected without difficulty.

After these experiments had been completed, a paper by Haarmann & Schroeder [1938] was published. These authors found increased formation of lactic acid from β-hydroxybutyric and dihydroxybutyric acids, both aerobically and anaerobically, with minced tissues of the dog and cat. On the basis of this result they put forward the very same mechanism of acetoacetic acid oxidation which I was led to reject as a result of my experiments. Though the possibility of a conversion of dihydroxybutyric acid into lactic acid is not denied and is indeed made probable by our experiments, the experiments of Haarmann & Schroeder are in my opinion open to certain criticisms. Haarmann & Schroeder write: "Die Dioxybuttersäure stört die Bestimmung der Milchsäure nicht." This statement cannot be accepted without a detailed description of the method employed to separate these acids. I have found that with the method of Friedemann *et al.* [1927], used by Haarmann & Schroeder (see Haarmann [1932]), up to 70 % is analysed as "lactic acid". This is in agreement with an observation of Friedemann [1928] who found 50 % "lactic acid" formed from dihydroxybutyric acid.

The copper-lime precipitation does not remove dihydroxybutyric acid quantitatively. Analysing a copper-lime filtrate I have still found 36% "lactic acid". With the periodic acid method it can be shown that only about 40% of the dihydroxybutyric acid is removed by the copper-lime treatment. Moreover, if the copper-lime precipitation were used, as was described by Haarmann in earlier work [1932], the lactic acid found may have been formed from acetol in a purely chemical, non-enzymic way, and evidence has been presented in this investigation that acetol is indeed thus formed from dihydroxybutyric acid. These points need clarifying before the evidence for the formation of lactic acid from dihydroxybutyric acid by tissues can be accepted.

It has already been pointed out that Haarmann & Schroeder's suggestion that dihydroxybutyric acid may be formed from acetoacetic acid by hydration is improbable on the ground that the process by which acetoacetic acid disappears anaerobically is a dismutation, i.e. the formation of equal amounts of reductive and oxidative equivalents. Since dihydroxybutyric acid is oxidized to hydroxyacetoacetic acid, the latter should also be formed by the dismutation of acetoacetic acid if the hydration mechanism operates; it has been shown that this is not the case.

These criticisms do not of course apply to the formation of lactic acid from $\beta$-hydroxybutyric acid described by Haarmann [1935] and by Haarmann & Schroeder [1938]. But it may be emphasized that in these experiments where a large formation of acetone takes place the preliminary removal of this substance before lactic acid estimation must be very thorough. Using this precaution I could not find any formation of lactic acid from acetoacetic acid with rat kidney slices.

## Summary

1. A method is described for the preparation of solutions containing equivalent amounts of $\alpha$-hydroxyacetoacetic and acetic acids by the anaerobic hydrolysis of ethyl $\alpha$-acetoxyacetoacetate in dilute alkali.

2. The stability of hydroxyacetoacetic acid in aqueous solution at *p*H 7·4 and 4·6 has been determined for 0, 25 and 37·5°.

3. Ethyl hydroxyacetoacetate is oxidized in $M/10$ $NaHCO_3$ by molecular $O_2$. 1 mol. $O_2$ is absorbed and 1 mol. acetic acid and 1 mol. ethyl hydrogen oxalate are formed.

4. Hydroxyacetoacetic acid is oxidized in $N/10$ NaOH by molecular $O_2$. 1 mol. $O_2$ is absorbed and 1 mol. $CO_2$, 1 mol. formic acid and 1 mol. acetic acid are formed. The latter appears as a polymerization product which is built up of the $CH_3$—CO— moiety of hydroxyacetoacetic acid and which yields acetic acid quantitatively after heating with acid.

5. The velocity of oxidation which is in many cases a measure of the tendency to enolization is of the following order: ethyl hydroxyacetoacetate > hydroxyacetoacetic acid > acetol. A number of oxidation reactions have been studied manometrically.

6. The estimation of acetol by reduction methods is described.

7. There is no effect on the respiration of rat tissues which could with certainty be attributed to hydroxyacetoacetic acid. A possibly oxidative removal is observed only with liver and heart. The only effect observed in presence of kidney slices is rapid decarboxylation.

8. If acetoacetic acid is incubated anaerobically with rat tissues a dismutation takes place, since the $\beta$-hydroxybutyric acid formed accounts only for 50% of the acetoacetic acid disappearing. But no evidence has been found which would

allow the identification of the oxidative equivalent with hydroxyacetoacetic acid.

9. *dl*-Threo-1:2-dihydroxybutyric acid is oxidized by slices of rat kidney to hydroxyacetoacetic acid. Its amount accounts for about 50% of the dihydroxybutyric acid disappearing. This proves that the failure to detect hydroxyacetoacetic acid amongst the oxidation products of acetoacetic acid is not due to a too rapid disappearance of hydroxyacetoacetic acid or to inadequate technique of detection.

I wish to acknowledge with gratitude the gift of samples of *dl*-threo- and erythro-1:2-dihydroxybutyric acids by Dr J. W. E. Glattfeld of the University of Chicago. My thanks are also due to Dr F. Dickens for his interest and encouragement.

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*Note added 26 May 1938.* Recent experiments indicate that the reducing substance found in the suspension fluid after aerobic incubation of rat kidney slices with acetoacetic acid is glucose.

# CXL. SPECTROGRAPHIC STUDIES ON THE ANTIMONY TRICHLORIDE REACTION FOR VITAMIN A

## II. INFLUENCE OF OXIDIZING AGENTS ON THE REACTION

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(*Received 26 April 1938*)

As mentioned in the first part of these studies [Notevarp & Weedon, 1936], the ratio between the two absorption bands of the antimony trichloride reaction for vitamin A is normally almost constant for potent preparations, but varies within wide limits for cod liver oils of usual potencies.

Mittelmann [1925; 1927], in publications reviewed by Bezssonoff [1929], claimed to have prepared cod liver oils giving no blue colour until some oxidation had taken place. Hawk [1929] obtained some increase in the blue value on exposure of cod liver oils to air. Lovern *et al.* [1931] were not able to prepare oils giving no reaction, but found a considerable rise in the 603 m$\mu$ band on oxidation of very fresh oils. The 572 m$\mu$ band remained approximately constant; this was confirmed by Heilbron *et al.* [1931]. We demonstrated earlier that very high concentrations of oil inhibited the 603 m$\mu$ band, but not the 572 m$\mu$ band, in fresh oils in which the 572 m$\mu$ band predominated.

In oils in which the maximum obtainable absorption has presumably been reached, the ratio of the 572 to the 603 m$\mu$ band is still far from constant. Morgan *et al.* [1935] found it to vary from 1·24 to 1·48 for 6 oils, Heilbron *et al.* [1931] from 1·09 to 1·77 for 15 oils. For concentrates of the same 6 oils Morgan *et al.* [1935] found from 1·72 to 2·06, while Carr & Jewell [1933] found 1·92 for vitamin A. We have found no published values for oils which approach the value for vitamin A.

As the 572 m$\mu$ absorption is little altered by the various factors that influence the reaction, it follows from the great variation of the ratio that the amount of inhibitor for the 603 m$\mu$ band must vary considerably. And as the ratio is always lower for oils than for concentrates, it may safely be assumed that the inhibition is never completely removed from reactions with oils by any of the means hitherto employed except saponification.

The very considerable inhibition shown by fresh oils is, as has been mentioned before, removed to a great extent by gentle oxidation, indicating that the inhibitor consists at least in part of an unsaturated substance. This may be the strongly inhibiting acid isolated by Emmerie [1932] in amounts of about 2·5 ml./l. from cod liver oil. Hydrogenation, resulting in total inactivation, required 4 atoms of hydrogen per mol.; calculated as oxygen this is about 7 mg./100 g. oil. According to Notevarp & Pillgram-Larsen [1933] this should give an increase in the Kreis value of 4–5 R.L.V., or a degree of oxidation which practically all commercial cod liver oils are subjected to during normal production operations. But of course it cannot be assumed *a priori* that the oxygen is absorbed by the inhibiting acid alone, although the unsaturation of the latter makes very easy oxidation probable.

In old or very rancid oils another kind of inhibition is seen, resulting in low or uncertain blue values for the oils, while their concentrates give clear reactions of normally high values.

The inhibition of the 603 m$\mu$ band would seem of considerable practical importance, in view of the great use made of the blue value commercially and scientifically for the determination of vitamin A. If no precautions are taken, very fresh or old oils will give values which lie far below those corresponding to the actual potency, and even after some oxidation has taken place the variations in the 603/572 m$\mu$ absorption ratio will indicate varying inhibition and therefore uncertain blue values. As will be shown later, the reagent may also provide a source of error, as some batches of it give higher values than usual with fresh oils.

These difficulties may presumably be overcome by carrying out the determinations on the unsaponifiable matter, as has been strongly advocated by a number of workers. For routine work, however, this is not practicable. In the time necessary for one determination on the unsaponifiable matter, it is possible to make a very large number of direct determinations. Also the preparation of the unsaponifiable fraction demands great skill and accuracy if the results are to be reliable, otherwise there is grave risk of increasing the error instead of reducing it.

The determination of the blue value of vitamin oils is today easily the most frequently used method of assaying vitamin A, and in view of the facts set out above, the accuracy and consistency of the values are surprisingly good. This must be due to the fact that it is chiefly the 603 m$\mu$ band that varies, and, as will be seen from Fig. 13 in the first part of this series, such variations influence the eye much less than variations near 572 m$\mu$, as long as the location of the absorption is unchanged.

Nevertheless, it would seem very desirable to be able to eliminate the variations in the 603 m$\mu$ absorption; this would mean a considerable increase in the reliability of the antimony trichloride reaction for vitamin A.

This study was started to investigate whether a definite maximum absorption at 603 m$\mu$ could be obtained by a definite amount of oxidation, by air or other means, so as to give better blue values without seriously increasing the complexity of the method.

## Experimental

### *Determination of the degree of oxidation*

To follow the degree of oxidation of the oils, we have used the Kreis test [cf. Taffel & Revis, 1931] as adapted to cod liver oils by Notevarp & Pillgram-Larsen [1932], which is shortly as follows.

The oil is shaken for 30 sec. with an equal volume of HCl, sp. gr. 1·19, in a small glass cylinder, internal diameter 20 mm.; 5 drops of phloroglucinol in alcohol, 50 g./l., are added, the mixture is shaken for a further 30 sec. and the resulting red colour is measured in a Lovibond Tintometer within 1 min. after the emulsion has separated. If the colour intensity is above 10 R.L.V. less oil is taken so as to get a colour below 10.

The other methods used have been described earlier.

### *Examination of oils at various stages of oxidation*

For these experiments we used cod liver oils taken from the boilers immediately after steaming and placed in hermetically sealed bottles. Many of the oils are those described in Part I of these studies, so the same numbers are used.

*First series.* Quantities of cod liver oil (no. 5, Part I) were shaken with varying volumes of air during several days, and examined after some weeks' storage. The results are seen in Table I and Fig. 1.

Table I

| Air/oil ratio | $E^{10\,g./l.}_{572\,m\mu}$ | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. 18·18 g./l. | Curve in Fig. 1 |
|---|---|---|---|---|
| 0 | 0·54 | 0·49 | 5·8 | I |
| 0·2 | 0·59 | 0·78 | 7·6 | II |
| 0·7 | 0·58 | 0·80 | — | — |
| 1·3 | 0·63 | 1·08 | — | III |
| 4·0 | 0·65 | 1·07 | 11·0 | IV |
| 2 years later 0·2 | 0·66 | 1·09 | 10·0 | — |

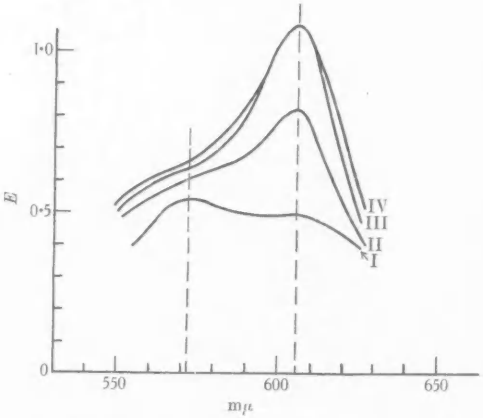


Fig. 1. Influence of oxidation of oil no. 5 on the absorption spectrum of the blue solution. I–IV rising intensity of oxidation, see Table I.

*Second series.* Another sample of cod liver oil (no. 4) was evacuated, heated to remove water, and cooled in a stream of $CO_2$. Quantities of this were again shaken with varying volumes of air. For the results see Table II.

Table II

| Air/oil ratio | Kreis value | $E_{572\,m\mu}$ | $E_{603 m\mu}$ | B.V. 18·18 g./l. |
|---|---|---|---|---|
| 0 | 3·4 | 0·55 | 0·49 | 5·8 |
| 0·5 | 11 | 0·50 | 0·45 | 5·5 |
| 1 | 14 | 0·51 | 0·61 | 5·6 |
| 4 | 46 | 0·51 | 0·76 | 6·4 |
| $1\frac{1}{2}$ years later 0·5 | — | 0·51 | 0·48 | 5·6 |
| 4 | — | 0·52 | 0·85 | 8·5 |

No definite maximum appeared to be reached even after considerable oxidation. The treatment of the oil might have influenced the result, so one more series was started.

*Third series. Oil no. 3.* Blue value and absorption rose, but the results do not allow any definite conclusions to be drawn (Table III). All three series show an

insignificant variation in the 572 mμ band, confirming what has been found by earlier workers. In the following the values for 572 mμ are therefore omitted.

Table III

| Air/oil ratio | Kreis value | $E_{572\,m\mu}$ | $E_{603\,m\mu}$ | B.V. 18·18 g./l. |
|---|---|---|---|---|
| 0 | 0·5 | 0·52 | 0·54 | 5·9 |
| 0 (heated) | 3·0 | 0·53 | 0·63 | 6·2 |
| 0·25 | — | 0·54 | 0·75 | 7·6 |
| 0·5 | — | 0·55 | 0·76 | 7·9 |
| 2·0 | 14 | 0·57 | 0·90 | 8·3 |
| (4·0 after 2 years | — | 0·42 | 0·65 | 6·8) |

*Fourth series.* Another sample (oil no. 2) was shaken with about an equal part of air at room temperature, and samples were drawn from the main bulk at intervals. For the results see Table IV.

Table IV

| Hours shaken | Kreis value | $E_{603\,m\mu}$ | B.V. 18·18 g./l. |
|---|---|---|---|
| 0 | 0·6 | 0·30 | 3·2 |
| 41 | — | 0·30 | 3·7 |
| 70 | 1 | 0·52 | 5·2 |
| 100 | 2·5 | 0·64 | 6·7 |
| 200 | 4·5 | 0·70 | 7·2 |
| Heated | 200 | 0·60 | 5·4 |

*Fifth series. Oil no. 1.* The heating accelerated the oxidation more than was intended, so a fifth experiment was run, in which the Kreis value was raised by prolonged shaking with air at room temperature.

Table V

| Kreis value | $E_{603\,m\mu}$ | B.V. 18·18 g./l. |
|---|---|---|
| 0·6 | 0·36 | 6·0 (1*a*) |
| 5·5 | 0·88 | 8·6 (1*b*) |
| 30 | 0·88 | 8·8 (1*c*) |

The last three series would seem to indicate that a degree of oxidation corresponding to a Kreis value somewhere about 10 is necessary to get the highest 603 mμ-absorption obtainable by direct oxidation with air. But the second series needed a Kreis value of nearly 50 before the maximum was reached. The only definite conclusion to be drawn is really that it would appear to be very difficult to find a useful analytical method based on oxidation with air.

### *Addition of oxidants to the reaction mixture*

The work of Heilbron *et al.* [1931] already mentioned led us to investigate the possibility of obtaining maximum absorption by the addition of chemical oxidants before or after the reagent had been added. Chlorine and antimony pentachloride dissolved in carbon tetrachloride were chosen, and measurements of the blue value of oil no. 1, the one which was used in series 5, were made when oxidants in varying concentrations were added. The results are collected in Table VI. The concentrations given are those in the reaction mixture; $SbCl_5$ is calculated as active chlorine.

Table VI

| Concentration of oxidant | B.V. 18·18 g./l. Cl addition Before | Cl addition After | $SbCl_5$ addition Before | $SbCl_5$ addition After |
|---|---|---|---|---|
| 0·01 | — | — | 8·8 | 8·0 |
| 0·02 | 9·5 | 7·0 | 8·8 | 8·3 |
| 0·05 | 9·4 | 8·0 | 9·5 | 9·4 |
| 0·10 | 9·0 | 9·0* | 9·5* | 9·1* |
| 0·20 | 9·0† | 9·1* | Violet, turning red | |

Unoxidized oil, ordinary reagent: B.V. = 6·0.
Max. obtainable with air B.V. = 8·8.

* Turning reddish very quickly. † Rapidly diminishing intensity.

Addition of chlorine before the reagent gave the highest values and seemed promising, so spectrographic measurements were made with oils nos. 1, 2 and 4. See Table VII for the results.

Table VII

| Oil no. | No addition of oxidant unoxidized oil $E_{603\,m\mu}$ | B.V. | Max. obtainable with air $E_{603\,m\mu}$ | B.V. | With Cl Cl g./l. | With Cl $E_{603\,m\mu}$ | With Cl B.V. |
|---|---|---|---|---|---|---|---|
| 1 | 0·36 | 6·0 | 0·88 | 8·8 | 0·02 | 0·70 | 8·1 |
| | | | | | 0·05 | 0·69 | 8·1 |
| 2 | 0·30 | 3·2 | 0·70 | 7·2 | 0·05 | 0·45 | 6·5 |
| 4 | 0·48 | 5·6 | 0·85 | 8·5 | 0·02 | 0·60 | 7·6 |

Both blue values and absorption rose, but the maximum values were definitely lower than those previously obtained by oxidation with air. This line of investigation was therefore not pursued further.

*Addition of oxidants to the reagent*

During work with series 3 it had been observed that some batches of reagent gave considerably higher 603 m$\mu$ absorption with fresh oils than normal. In determining the blue values of fresh oils we had often observed earlier that the values might vary with different batches of reagent. Attempts were made to determine the oxidizing properties of various batches by distillation with acid KI solution, and iodine was found to be liberated in amounts corresponding to 0·1–0·6$\gamma$ $O_2$/ml. reagent, i.e. about 0·5–3 mg. $O_2$/100 g. oil, in a reaction mixture of normal concentration (18·18 g./l.). This is of the same magnitude as the figure arrived at earlier, but the results were not consistent. At that time our major concern was to ascertain the influence of other oxidants, so in order to be certain of eliminating oxidation caused by the reagent, special care was taken to obtain reagents free from oxidizing substances. The antimony trichloride proved not to contain appreciable amounts of pentavalent antimony, so the oxidation must presumably have been due to decomposition of the chloroform.

These observations proved the possibility of oxidation by means of the reagent, but its spontaneous oxidizing properties could not be produced at will. The next step was therefore to try adding oxidizing substances to normal reagents. Various oxidants were tried: chlorine, which may safely be assumed to form pentachloride immediately, also antimony pentachloride as such, and bromine. Test tube experiments showed that a considerable amount of bromine can be added to the reagent before the brown colour persists, indicating the formation of mixed chloride-bromides of pentavalent antimony. Iodine does not

form mixed halogenides in this way, as there is no reduction in the depth of violet colour.

The use of chlorine, antimony pentachloride or bromine proved successful, as higher blue values were obtained than by any other means. Preliminary experiments were made to find the best oxidant and the most suitable concentration (see Fig. 2).

The maximum obtainable blue value was about the same for the three oxidants. Antimony pentachloride gave two slightly higher values, but these results have not since been confirmed, so they can probably be put down as experimental errors.

The results clearly showed that bromine gives a maximum blue value over a much wider range of concentration than chlorine. The minimum concentration necessary is about 0·02 g./l. or less, for all three oxidants.

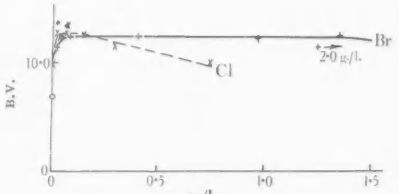


Fig. 2.

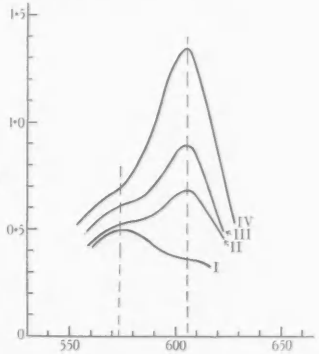


Fig. 3.

Fig. 2. *Blue values of oil no. 1* with varying concentrations of oxidants in the reagent. o Ordinary reagent. • $SbCl_5$ reagent. × Chlorine reagent. + Bromine reagent.

Fig. 3. *Absorption curves of oil no. 1* unoxidized and oxidized by various means. I, unoxidized. II, chlorine added before reagent. III, maximum absorption after oxidation with air. IV, unoxidized oil with bromine reagent.

The rise in absorption was then measured spectrographically. In Table VIII are collected the results for three quite fresh oils using reagents containing different oxidants; in the case of one of the oils, the results at two stages of oxidation with air are given as well. For comparison are also given values for the unoxidized oils and for the oils after oxidation by the addition of chlorine and by air.

Table VIII

| Oil no. | No addition | | Cl before addition of reagent | | Max. obtainable with air | | Addition to reagent: $Cl_2$ | | $SbCl_5$ | | $Br_2$ | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. | $E^{10\,g./l.}_{603\,m\mu}$ | B.V. |
| 1*a* | 0·36 | 6·0 | 0·70 | 8·1 | 0·88 | 8·8 | 1·23 | 13·0 | 1·25 | 14·0 | 1·35 | 13·8 |
| 1*b* | 0·88 | 8·6 | — | — | — | — | — | — | — | — | 1·32 | 12·0 |
| 1*c* | 0·88 | 8·8 | — | — | — | — | 1·05 | 10·3 | — | — | 1·10 | 10·5 |
| 2 | 0·30 | 3·2 | 0·45 | 6·5 | 0·70 | 7·2 | — | — | 0·93 | 9·4 | 0·94 | 9·4 |
| 4 | 0·48 | 5·6 | 0·60 | 7·6 | 0·85 | 8·5 | — | — | — | — | 1·23 | 10·8 |

The result is striking; this is shown clearly in Fig. 3 where the complete curves for oil no. 1 are shown.

Addition of oxidants to the reagent results in higher 603 mμ absorption values than can be obtained by any other means, and these values are obtained even with unoxidized oils, which with the ordinary reagent give higher absorption at 572 mμ than at 603 mμ. One oil gave the same maximum with such reagents both unoxidized and after mild oxidation, which makes it probable that a definite maximum was reached. On the other hand, it appears that the 572 mμ absorption does not change more than can be ascribed to overlapping of the absorption bands.

Bromine gives slightly higher values than chlorine or antimony pentachloride, but the differences do not seem significant. Bromine, however, gives maximum blue value over far the greatest range of concentration, and is also the easiest to obtain and handle, so bromine has been chosen as the oxidizing reagent.

*Optimum concentration of bromine.* $E_{603\,m\mu}$ was measured with three different concentrations of Br in the reagent. The results are seen in Table IX.

Table IX

| | Concentration of Br, g./l. | | | |
|---|---|---|---|---|
| | 0 | 0·05 | 0·10 | 0·50 |
| Oil no. | $E^{10\,g./l.}_{603\,m\mu}$ | $E^{10\,g./l.}_{603\,m\mu}$ | $E^{10\,g./l.}_{603\,m\mu}$ | $E^{10\,g./l.}_{603\,m\mu}$ |
| 15 | 0·21 | 0·30 | 0·32 | 0·32 |
| 3 fresh, Kreis 0·5 | 0·54 | 1·08 | 1·08 | 1·08 |
| 3 oxidized, 2 years' old | 0·65 | 0·91 | 0·95 | 0·99 |
| 18 (old, oxidized) | 17·8 | 19·0 | 18·3 | 19·0 |
| 14 Halibut liver oil Kreis 600 | 163 | 168 | 174 | 174 |

There seems to be a tendency towards rising values with rising concentrations for old and oxidized oils; for fresh oils no such tendency is apparent over the range tried.

The smallness of the rise in absorption with concentrations exceeding 0·1 g./l. suggests that higher concentrations are not necessary, so this concentration was chosen. For practical purposes it is unnecessary to measure the amount of bromine with any great accuracy, as variations in the concentration have no very marked effect. One drop of bromine to 1 l. of reagent corresponds to about 0·1 g./l.

*Relative intensities of the absorption bands with bromine reagent*

In Table X we have collected values for the 572 and 603 mμ maxima obtainable with air, and with bromine reagent.

Table X

| | $E_{572\,m\mu}$ | | $E_{603\,m\mu}$ | | Ratio | |
|---|---|---|---|---|---|---|
| Oil no. | Air | Br | Air | Br | Air | Br |
| 15 | 0·14 | 0·19 | 0·21 | 0·32 | 1·50 | 1·69 |
| 2 | 0·45 | 0·50 | 0·70 | 0·94 | 1·55 | 1·88 |
| 19 | — | 0·60 | — | 1·06 | — | 1·77 |
| 3 | 0·55 | 0·60 | 0·96 | 1·08 | 1·74 | 1·80 |
| 4 | 0·52 | 0·65 | 0·85 | 1·23 | 1·63 | 1·90 |
| 1 | 0·60 | 0·70 | 0·88 | 1·35 | 1·47 | 1·93 |
| 20 | — | 0·85 | — | 1·60 | — | 1·88 |
| 21 | — | 1·0 | — | 1·80 | — | 1·80 |
| Vitamin A (Carr & Jewell) | 2600 | | 5000 | | 1·92 | |

No great accuracy is claimed for the determinations of the 572 mμ absorption, but the trend of the values seems unmistakable. The average ratio is considerably higher than that found by previous workers using the ordinary reagent, and approaches that of Carr & Jewell for vitamin A, which is included in Table X for comparison.

*Tintometer readings and spectral absorption with bromine reagent*

Tintometer readings and $E$ values obtained by the use of bromine reagent (0·1 g./l.) are given in Table XI, and plotted graphically in a bi-logarithmic co-ordinate system in Fig. 4 (cf. Table II and Fig. 5 in Part I of these studies, Notevarp & Weedon [1936]).

Table XI

| Oil no. | $E_{603\,m\mu}$ | B.V. | Oil no. | $E_{603\,m\mu}$ | B.V. |
|---|---|---|---|---|---|
| 1*a* | 2·26 | 12·8 | 16 | 3·09 | 15·5 |
| | 1·13 | 8·0 | | 1·62 | 10·5 |
| 1*b* | 2·20 | 11·0 | | 0·83 | 6·0 |
| | 1·10 | 6·5 | 17 | 3·63 | 14·5 |
| 1*c* | 1·87 | 10·0 | | 1·90 | 9·5 |
| | 0·98 | 6·3 | | 0·97 | 6·2 |
| 2*a* | 2·23 | 11·0 | 19 | 2·70 | 12·5 |
| | 1·61 | 9·0 | | 1·95 | 10·0 |
| | 1·54 | 8·5 | | 1·02 | 6·7 |
| 3*a* | 1·76 | 10·8 | | 1·49 | 9·1 |
| | 0·92 | 6·1 | | 0·72 | 4·7 |
| 3*b* | 1·55 | 8·8 | 20 | 2·61 | 14·0 |
| 4*a* | 2·88 | 13·0 | | 1·36 | 8·7 |
| | 1·37 | 10·7 | | 0·78 | 5·3 |
| | 1·05 | 6·2 | | 0·70 | 4·8 |
| 15 | 1·68 | 8·5 | 21 | 2·94 | 15·5 |
| | 1·17 | 6·5 | | 1·54 | 9·4 |
| | 0·61 | 4·0 | | 0·86 | 6·0 |
| | | | | 0·79 | 5·8 |

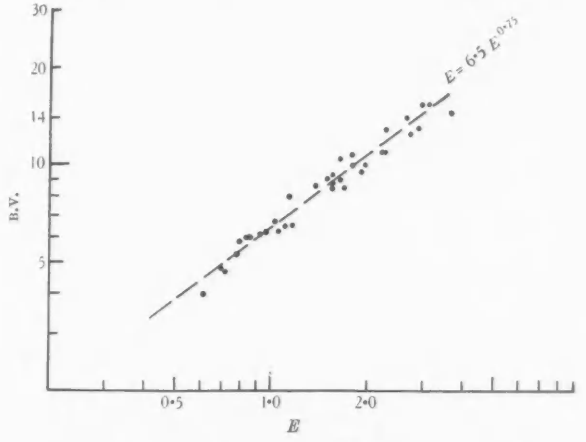


Fig. 4. B.V. and $E$ for cod liver oils with bromine reagent.

Fig. 4 shows that the function B.V. $= 6{\cdot}5\ (E_{603\,m\mu})^{0{\cdot}75}$ corresponds to the average of the points found. The spreading of the values is about the same as with the ordinary reagent. As has been said before, this spreading is probably chiefly due to the inherent inaccuracies of the determination of blue values.

## Discussion

The results obtained during these studies show that it is apparently very difficult to remove from fresh cod liver oils the factors inhibiting the 603 m$\mu$ band either by controlled oxidation with air or by addition of oxidants such as chlorine, antimony pentachloride or bromine before or after adding the reagent.

If, however, these oxidants are added to the reagent itself in minute quantities, a very definite increase in the 603 m$\mu$ band is obtained. The absorption maximum becomes constant and independent of the degree of oxidation up to the point at which partial destruction of the chromogen becomes highly probable; it is also independent of the concentration of oxidant in the reagent over a varying range. The absorption is also increased by such reagents in the case of old and oxidized oils. In all oils there is an increase in blue value and absorption beyond those which can be obtained with the ordinary reagent.

The ratio of the 603 m$\mu$ band to the 572 m$\mu$ band increases and approaches that found for pure vitamin A. The variation is probably no more than the experimental error, even for oils which with ordinary reagent show a predominance of the 572 m$\mu$ band.

These facts all indicate that the addition of oxidants to the reagent removes a very considerable portion of the inhibiting influences found in cod liver oil, even such inhibition as is not removed by gentle oxidation of the oil. The constancy of the maximum, independently of the degree of oxidation or of the concentration of oxidant, also makes it probable that the inhibition is completely or nearly completely removed. Whether this is the case can be ascertained by comparison of the absorption of oils with that of their concentrates, which will be discussed in Part III of these studies.

Bromine gives slightly higher values than the other oxidants that have been tried; it also gives maximum absorption over a much wider range of concentration. This and the accessibility of this substance make it natural to choose bromine as the standard oxidant for the new type of reagent.

The relation between $E$ and B.V. follows a similar exponential curve to that for the ordinary reagent. The alteration of the 603/572 m$\mu$ ratio increases the constant by about one unit, and also makes the slope of the curve slightly steeper, corresponding to an exponent of about 0·75.

## Summary

A definite maximum of the 603 m$\mu$ band of the antimony trichloride reaction with cod liver oils could not be obtained either by controlled oxidation or by the addition of oxidants before or after adding the reagent.

Addition to the reagent of small quantities of oxidants such as chlorine, bromine or antimony pentachloride resulted in higher 603 m$\mu$ absorption values than were obtained by any other method for oils in all stages of oxidation ranging from quite fresh ones, with the 572 m$\mu$ band predominating when the ordinary reagent is used, to very old or rancid oils.

The ratio of $E_{603\,m\mu}$ to $E_{572\,m\mu}$ increases, becomes more constant and approaches that of pure vitamin A preparations, 1·92.

It is probable that the inhibition of the 603 m$\mu$ band is completely or nearly completely removed by the oxidizing reagent.

Bromine gives the highest absorption of the agents that have been tried; it is also the most generally accessible. 0·1 g./l. is a suitable concentration.

With this reagent the relation between B.V. and $E_{603\,m\mu}$ follows the equation:

$$\text{B.V.} = 6{\cdot}5\,(E_{603\,m\mu})^{0{\cdot}75}.$$

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# CXLI. GLUTAMIC ACID DEHYDROGENASE FROM GERMINATING SEEDS[1]

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Little definite knowledge exists as to the mechanism of oxidative deamination in plants. That such deamination does occur is to be inferred both from the values of the respiratory quotients of certain germinating seeds [Malhotra, 1933] which indicate protein catabolism, as well as from the well-known accumulation of asparagine and glutamine during germination which has been shown to take place only in the presence of oxygen [Palladin, 1889]. As the amount of asparagine found is usually far in excess of what can be accounted for on the basis of the aspartic acid content of the seed proteins and, further, as the increase in asparagine is concomitant with the disappearance of other amino-acids [Schulze, 1898; 1901] there can be no doubt that its formation takes place at the expense of the latter. It is generally assumed that the ammonia formed by oxidative deamination condenses with $COOH.CO.CH_2.COOH$ arising either from carbohydrates or from deaminized amino-acid residues to give first aspartic acid and then its amide. In the case of glutamine the position is rather different on account of the high content of the seed proteins in glutamic acid. As Greenhill & Chibnall [1934] have pointed out, with the evidence at present available it is equally valid to assume either that the glutamine present in the seed is the direct product of the hydrolysis of protein [Damodaran *et al.* 1932] or that it arises from the combination of ammonia with a non-nitrogenous residue.

One of the most interesting results of recent work on the oxidation of amino-acids in animal tissues is the hypothesis due to Krebs [1935, 1, 2] that in kidney tissue glutamine synthesis takes place through the intervention of a "deaminase". The disappearance of ammonia during the oxidation of glutamic and aspartic acids by muscle was noted by Needham [1930]. Krebs observed a similar effect with glutamic acid in the presence of kidney slices; he was able to show that the disappearance of ammonia was accompanied by an increase of amide-N determined by the method of Sachsse [1873] and further that the disappearance of added ammonia could be brought about by kidney slices in the presence of α-ketoglutaric acid. These observations taken in conjunction with the existence of a glutaminase in kidney have led Krebs to the inference that in the intact organ the deaminase system studied by him is really concerned, not with the oxidation of glutamic acid, but with the reverse synthetic process, viz. the formation of glutamine from ammonia and ketoglutaric acid, the latter probably arising from carbohydrate oxidation via pyruvic acid and αα-diketoadipic acid [Toenniesen & Brinkmann, 1930].

It is of obvious interest to ascertain if this very attractive hypothesis is applicable to the vegetable organism also, especially in view of the fact that the experimental evidence for the formation of glutamine adduced by Krebs rests

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solely upon the determination of amide-N by acid hydrolysis, a method which can hardly be called specific [cf. Chibnall & Westall, 1932], whereas the formation of glutamine in the plant has been proved beyond doubt by actual isolation by a number of workers.

The present paper describes in the first place experiments carried out to ascertain the existence of deaminases in germinating seeds and secondly the properties of the only amino-acid dehydrogenase which was actually found in the seedlings examined, viz. one specific for glutamic acid.

Maceration extracts of the seedlings of about a dozen species (mostly of the leguminous family) were tested, by the methylene blue and the manometric methods, for their actions on the following amino-acids: glycine, alanine, leucine, tyrosine, histidine, aspartic acid, glutamic acid and *dl*-proline. Glutamic acid was the only amino-acid that was acted upon and even this action was found to be limited to three out of the twelve species examined, viz. to *Phaseolus mungo*, *P. radiatus* and *Pisum sativum*. In being obtained in cell-free extracts capable of oxidizing the natural *l*(+)glutamic acid the enzyme found in germinating seeds differs from the deaminases studied by Bernheim and coworkers [1934, 1, 2; 1935, 1, 2] by Krebs [1933, 1, 2; 1934; 1935, 1, 2, 3] and by Weil-Malherbe [1935; 1936] whose preparations in extracts free from cellular material acted only on the optically non-natural amino-acids. Purification of the dehydrogenase by adsorption on kaolin, kieselguhr and aluminium hydroxide proved unsuccessful. Some concentration could be effected by precipitation of the maceration extract by saturation with ammonium sulphate. But the precipitate thus obtained could not be freed from ammonium sulphate as dehydrogenase activity was completely lost during dialysis. For all experiments, therefore, the ammonium sulphate precipitate redissolved in phosphate buffer at *p*H 7·8 and without dialysis was used. Seedlings of *Phaseolus mungo* two days old were found to be the best starting material. The initial oxygen uptakes of such preparations were quite low being generally about 50 $\mu$l./hr./ml. The enzyme was always contaminated with peroxidase. It could, however, be readily demonstrated by means of differential inactivation that peroxidase activity was in no way responsible for the oxidation of glutamic acid. Thus treatment with acetone had very little influence on peroxidase (as determined quantitatively by the oxidation of Nadi reagent) while glutamic acid dehydrogenase was completely destroyed by the process.

Being capable of utilizing either oxygen or methylene blue as hydrogen acceptor the enzyme belongs to the class of oxytropic dehydrogenases. The action in the presence of oxygen is not inhibited by KCN up to a concentration of 0·005 *M* or by the other inhibitors tried, viz. arsenite and fluoride. The following substances were tried on the enzyme system to determine if they could function as hydrogen transporters in the reaction: methylene blue, ascorbic acid, glutathione and dihydroxyphenylalanine. None of these were found to have any accelerating effect on the oxygen consumption.

Inactivation by alcohol precluded the use of the method based on the coupled oxidation of alcohol by which Keilin & Hartree [1936] had demonstrated the formation of hydrogen peroxide in the case of kidney deaminase. The "Abfangsverfahren" of Wieland & Rosenfeld [1930] using ceric sulphate also gave negative results. But taking advantage of the presence of peroxidase in the enzyme preparation used, production of hydrogen peroxide could be demonstrated by the coupled oxidation of nitrite [Thurlow, 1925].

The oxygen uptake corresponds to one atom of oxygen to each molecule of glutamic acid. The end product of the reaction was shown to be $\alpha$-ketoglutaric

acid which was isolated and identified as the dinitrophenylhydrazone. The reaction taking place is therefore as in the case of kidney deaminase:

$$COOH.CH_2.CH_2.CH(NH_2).COOH + \tfrac{1}{2}O_2 \rightarrow COOH.CH_2.CH_2.CO.COOH + NH_3.$$

The production of ammonia could not, however, be shown as exact ammonia determinations were rendered impossible by the presence of ammonium sulphate in the enzyme preparation used.

## Experimental

### *Methods*

Qualitative data such as the distribution of the dehydrogenase in seedlings were obtained by Thunberg's methylene blue technique; tubes of the Keilin type were used. In the use of Barcroft's manometer for the measurement of oxygen uptake the instructions given by Dixon [1934] were followed in detail. The manometers were calibrated both by calculation and by Munzer & Neumann's method; the values of the manometric constant obtained by both methods were in close agreement. 0·4 ml. of a 40 % solution of sodium hydroxide and a small roll of No. 42 Whatman filter paper were placed in the centre compartment of the manometric cup. The rate of shaking was kept at 120–180 oscillations per minute according to the rate of oxygen uptake. The substrate was placed in small Keilin cups and added to the reaction mixture after equilibration.

For the estimation of peroxidase a method due to Guthrie [1931] based upon the colorimetric measurement of the amount of indophenol formed in a definite time from a mixture of *p*-phenylenediamine and α-naphthol in the presence of hydrogen peroxide was used. The reagent was made up by adding to 200 ml. of citrate buffer at *p*H 4·5 200 ml. of water, 1 g. of *p*-phenylenediamine hydrochloride and 20 ml. of a 4 % solution of naphthol in 50 % alcohol and filtering. To 25 ml. of this in a centrifuge tube were added 0·5 ml. of the solution to be tested and 5 ml. of a 0·1 % solution of KCN. The mixture was shaken up with toluene, centrifuged and the solution of the indophenol in toluene compared in the colorimeter with an artificial standard consisting of 1 g. of iodine in 500 ml. of chloroform.

The amino-acids used were analytically pure samples prepared in the laboratory with the exception of *dl*-proline which was obtained from Messrs Hoffmann La-Roche. The dibasic amino-acids were neutralized with NaOH before use.

### *Presence of amino-acid dehydrogenase in seedlings*

The seedlings were washed and weighed after removal of the testa. They were ground up for 15 min. with half the weight of 0·87 % $K_2HPO_4$ and a little acid-washed sand, pressed through muslin and centrifuged. The supernatant was tested for amino-acid dehydrogenases using in every case 0·5 ml. of the extract to be tested, 0·2 ml. of an *M*/10 solution of the amino-acid, 0·3 ml. of 1/5000 methylene blue and 2 ml. of *M*/15 phosphate buffer at *p*H 7·8. Each tube had two controls, one with the boiled extract and the other with no amino-acid added.

Table I gives the results with different seedlings using various amino-acids. Glutamic acid is seen to be the only one of the amino-acids which accelerates the reduction of methylene blue. Even in this the action is confined to three of the seedlings examined, namely *Phaseolus mungo*, *P. radiatus* and *Pisum sativum*.

Where glutamic dehydrogenase is present a feeble action on alanine also is noticeable; but this seems to be too small to be of significance; the Thunberg factor [Thunberg, 1920] is below 2.

Table I. *Time for decoloration of methylene blue in Thunberg tubes*

(Figures in brackets refer to substrate-free controls. In boiled controls the time was the same in all cases.)

| | Time in minutes | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Seedling tried | Glycine | Alanine | Tyrosine | Histidine | Aspartic acid | Leucine | Glutamic acid | *dl*-Proline |
| *Phaseolus mungo* | 23 (23) | 17 (21) | 28 (28) | 28 (28) | 19 (19) | 23 (24) | 6 (18) | 22 (22) |
| *Phaseolus radiatus* | 12 (12) | 10 (12) | 12 (12) | 16 (16) | 13 (12) | 9 (10) | 5 (11) | 13 (12) |
| *Dolichos lab lab* | 22 (22) | 25 (25) | 25 (25) | 30 (29) | 28 (28) | 23 (23) | 23 (23) | 23 (23) |
| *Dolichos biflorus* | No decoloration in 2 hr. | | | | | | | |
| *Cicer arietinum* | 6 (6) | 6 (6) | 8 (8) | 8 (8) | 9 (8) | 8 (8) | 5 (6) | 6 (6) |
| *Canavalia ensiformis* | 35 (35) | 27 (27) | 27 (27) | 32 (33) | 33 (33) | 30 (31) | 24 (25) | 26 (25) |
| *Canavalia obtusifolia* | 31 (30) | 33 (33) | 41 (41) | 41 (41) | 37 (36) | 36 (36) | 32 (33) | 38 (36) |
| *Pisum sativum* | 22 (22) | 18 (19) | 23 (22) | 22 (22) | 19 (19) | 19 (19) | 8 (19) | 21 (22) |
| *Citrillus colocynthis* | 82 (85) | 78 (76) | 82 (85) | 76 (76) | 78 (76) | 81 (82) | 80 (82) | 69 (69) |
| Coconut | No decoloration in 2 hr. | | | | | | | |
| Wheat | No decoloration in 2 hr. | | | | | | | |
| *Citrillus vulgaris* | No decoloration in 2 hr. | | | | | | | |

*Purification of the enzyme*

Precipitation with dilute acetic acid, sodium sulphate and acetone and electrodialysis were tried as methods of purification, but all the methods yielded inactive preparations. The enzyme was not adsorbed on kaolin, kieselguhr or aluminium hydroxide. Precipitation by saturation with ammonium sulphate and taking up in phosphate buffer at *p*H 7·8 yielded an active preparation with a low initial oxygen uptake.

Seedlings between 2 and 3 days' old yielded the most active preparation. 20 ml. of the extract (*p*H 6·1) were cooled in ice and brought to *p*H 5·2 by the addition of very dilute acetic acid. After saturation with ammonium sulphate and centrifuging for 10 min. at 2000 r.p.m. the clear supernatant liquid was discarded. The residue was washed with phosphate buffer at *p*H 5·2 and redissolved in phosphate buffer at *p*H 7·8. The preparation in the absence of glutamic acid did not appreciably reduce methylene blue and had an oxygen uptake of about 50 $\mu$l./hr./ml.

*Differentiation from peroxidase activity*

Peroxidase was invariably found in the seedlings and persisted in the preparation obtained as described above. Previous attempts at purification had shown that drying with acetone destroyed dehydrogenase activity. A comparison was therefore made of the effects of acetone treatment on the peroxidase activity and on the oxidation of glutamic acid.

20 g. of the seedlings were extracted with 20 ml. 0·87 % $K_2HPO_4$. The extract was poured slowly with stirring into five times the volume of acetone cooled in ice. The precipitate was washed on the centrifuge with 5 ml. of acetone, spread out on a Petri dish and the acetone driven off by aeration. The yellowish white powder so obtained was suspended in 20 ml. of phosphate buffer at *p*H 7·8

and the activities of the dehydrogenase and peroxidase determined by the manometric method and by Guthrie's method respectively. For comparison the same determinations were done on the fresh extract of the seedlings. The results of three different experiments are given in Table II.

Table II. *Effects of acetone treatment on dehydrogenase and peroxidase*

| Peroxidase: Length of column in colorimetric tube (standard at 20 mm.) | | | Dehydrogenase: Oxygen uptake with glutamic acid ($\mu$l./hr.) | | |
|---|---|---|---|---|---|
| Before | After | % peroxidase | Before | After | % dehydrogenase |
| 18·2 | 26·1 | 76·6 | 238·2 | 0 | 0 |
| 29·5 | 38·9 | 76·0 | 250·0 | 0 | 0 |
| 10·2 | 13·1 | 77·7 | 242·0 | 0 | 0 |

The results clearly indicate that the oxidation of glutamic acid is unconnected with peroxidase. Though more than 75 % of the peroxidase remained after acetone-drying the manometric oxygen uptake was zero.

*Variation of activity with* p*H*

The variation of the oxygen uptake with *p*H is illustrated in Fig. 1. Below *p*H 5·9 the enzyme is totally inactive. The optimum *p*H is 7·8–8·0. The reduction of methylene blue in Thunberg tubes does not appear to be so susceptible to changes in *p*H as the oxygen uptake (Table III); at *p*H 5·9, however, the reduction of methylene blue also ceases.

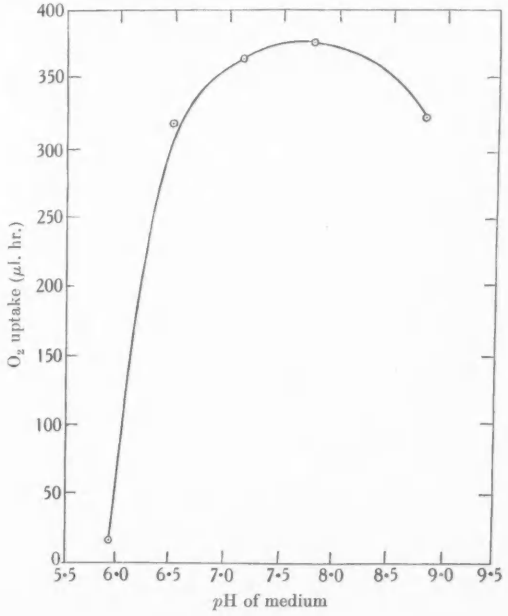


Fig. 1.

Table III. *pH and time of decoloration of methylene blue*

| No. | *p*H of medium | Time of decoloration |
|---|---|---|
| 1 | 5·9 | ∞ |
| 2 | 6·5 | 23 min. |
| 3 | 6·8 | 23 min. |
| 4 | 7·2 | 24 min. |
| 5 | 7·8 | 23 min. |
| 6 | 8·7 | 24 min. |

*Optimum substrate concentration*

Fig. 2 shows the effect on the rate of oxygen uptake and Table IV on the time for reduction of methylene blue of variations in glutamic acid concentration. 0·045 *M* is the optimal substrate concentration as determined from the graph.

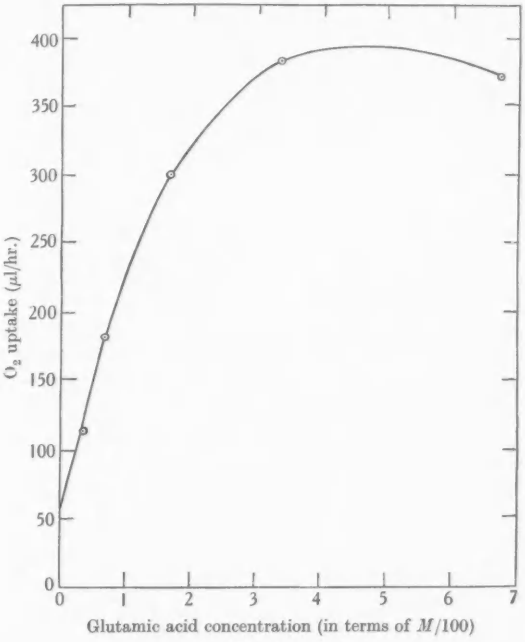


Fig. 2.

Table IV. *Decoloration of methylene blue and substrate concentration*

| Tubes ... | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Enzyme | 0·5 ml. | 0·5 ml. | 0·5 ml. | 0·5 ml. | 0·5 ml. | 0·5 ml. |
| Methylene blue 1/5000 | 0·3 ml. | 0·3 ml. | 0·3 ml. | 0·3 ml. | 0·3 ml. | 0·3 ml. |
| Glutamic acid *M*/10 | 2·0 ml. | — | — | — | — | — |
| Glutamic acid *M*/20 | — | 1·0 ml. | 0·5 ml. | — | — | — |
| Glutamic acid *M*/100 | — | — | — | 1·0 ml. | 0·5 ml. | — |
| Buffer *p*H 7·8 | 1·2 ml. | 2·2 ml. | 2·7 ml. | 2·2 ml. | 2·7 ml. | 3·2 ml. |
| Time for decoloration | 19 min. | 24 min. | 31 min. | 39 min. | 42 min. | 127 min. |

*Inactivation by heat*

10 ml. of the enzyme solution were placed in each of several test tubes kept immersed for different periods in baths at different temperatures. The solutions were cooled and the dehydrogenase activity in each tested manometrically. Slight inactivation was evident even at 37°. At 50° and above the destruction of the enzyme was very rapid (Table V).

Table V. *Inactivation by heat*

| Time of heating min. | Temperatures 37° µl. | 40° µl. | 50° µl. | 60° µl. | 70° µl. | 80° µl. |
|---|---|---|---|---|---|---|
| 5 | 362·0 | 352·5 | 182·5 | 17·5 | 0 | 0 |
| 30 | 341·0 | 245·0 | 0 | 0 | 0 | 0 |
| 60 | 328·5 | 97·5 | 0 | 0 | 0 | 0 |

*Formation of hydrogen peroxide*

Fixation of hydrogen peroxide by oxidation of cerium hydroxide to cerium peroxide [Wieland & Rosenfeld, 1930] and determination of the cerium peroxide by permanganate titration failed to yield any clear-cut evidence of the formation of hydrogen peroxide. The coupled oxidation of alcohol was also inapplicable as the alcohol concentration used by Keilin & Hartree [1936] was found to retard the dehydrogenase action. It was found, however, that the method used by Thurlow [1925] for xanthine oxidase, based on the oxidation of nitrite to nitrate by peroxidase at the expense of the hydrogen peroxide formed by dehydrogenation, could be applied here on account of the presence of peroxidase in the enzyme preparation. The enzyme was allowed to act upon glutamic acid in the presence of added sodium nitrite, the reaction mixture being aerated for a definite period. At the end the solutions were centrifuged and the amount of unoxidized nitrite determined colorimetrically using Griess-Ilosvay's reagent. Table VI gives the composition of the reaction mixture and the colorimetric readings in a typical experiment in which the time of aeration was 45 min.

Table VI. *Coupled oxidation of nitrite*

| Flasks ... ... | 1 | 2 | 3 |
|---|---|---|---|
| Enzyme | 10 ml. | 10 ml. | — |
| Glutamic acid $M/10$ | 5 ml. | — | 5 ml. |
| Sodium nitrite 0·02% | 2 ml. | 2 ml. | 2 ml. |
| Buffer $p$H 7·8 | — | 5 ml. | 10 ml. |
| Unoxidized nitrite (as length of column in colorimetric tube to balance standard set at 5 mm.) | 35·5 mm. | 6·2 mm. | 5·1 mm. |

*Effect of various hydrogen transporters*

Appropriate experiments showed that methylene blue, ascorbic acid, glutathione and dihydroxyphenylalanine were incapable of acting as intermediate carriers in the oxidation of glutamic acid by the dehydrogenase. In no case was a significant acceleration of oxygen uptake observed on the addition of these substances.

*Inhibiting agents*

KCN up to 0·005 $M$ had little effect on the oxidation (Table VII and Fig. 3). With higher concentrations the rate fell appreciably but the oxygen consumption was not completely inhibited even by a cyanide concentration of 0·2 $M$. Arsenite

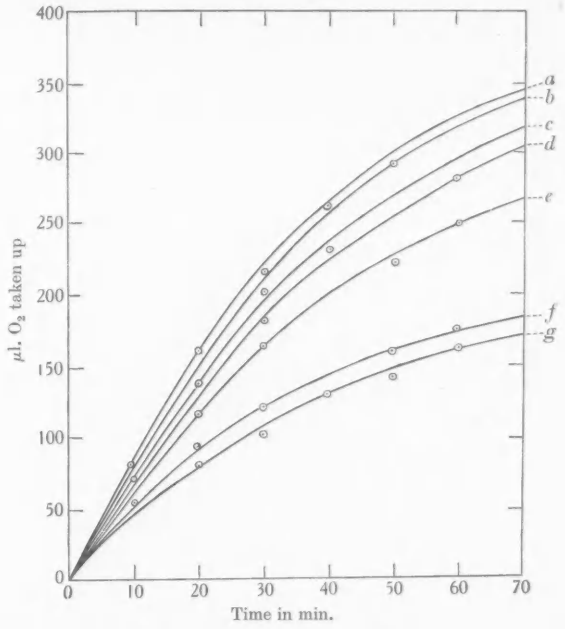


Fig. 3. $a$=without (CN); $b$=0·001 $M$ (CN); $c$=0·002 $M$ (CN); $d$=0·005 $M$ (CN); $e$=0·01 $M$ (CN); $f$=0·1 $M$ (CN); $g$=0·2 $M$ (CN).

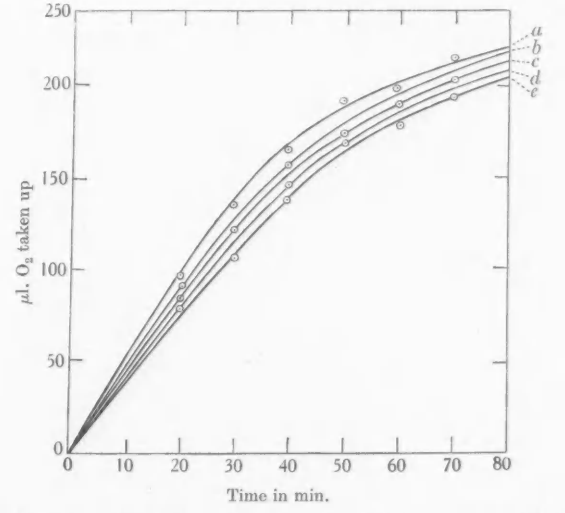


Fig. 4. $a$ and $b$=no arsenite; $c$=0·001 $M$ arsenite; $d$=0·002 $M$ arsenite; $e$=0·02 $M$ arsenite.

(Fig. 4) and fluoride in the concentrations generally employed did not lower the oxygen consumption to any extent. According to the accepted ideas this would indicate that oxygen activation is not involved in the action of the enzyme.

Table VII. *Effect of potassium cyanide on the $O_2$ uptake*

| Manometers ... | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|---|---|---|---|---|---|---|---|---|
| Enzyme | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. |
| Glutamic acid | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. | 1·0 ml. |
| Cyanide *M* | 0·6 ml. | 0·3 ml. | — | — | — | — | — | — |
| Cyanide 0·1 *M* | — | — | 0·6 ml. | 0·3 ml. | — | — | — | — |
| Cyanide 0·05 *M* | — | — | — | — | 0·3 ml. | — | — | — |
| Cyanide 0·01 *M* | — | — | — | — | — | 0·6 ml. | 0·3 ml. | — |
| Buffer *p*H 7·8 | 0·4 ml. | 0·7 ml. | 0·4 ml. | 0·7 ml. | 0·7 ml. | 0·4 ml. | 0·7 ml. | 1·0 ml. |
| $O_2$ uptake ($\mu$l./hr.) | 97·2 | 115·0 | 117·0 | 196·0 | 251·5 | 270·0 | 298·0 | 296·0 |

*Amount of oxygen utilized per molecule of glutamic acid*

The rate of oxygen uptake of the enzyme during the complete oxidation of 1 ml. of *M*/50 glutamic acid was studied. The completion of the oxidation was shown by the cessation of the oxygen consumption (Table VIII and Fig. 5).

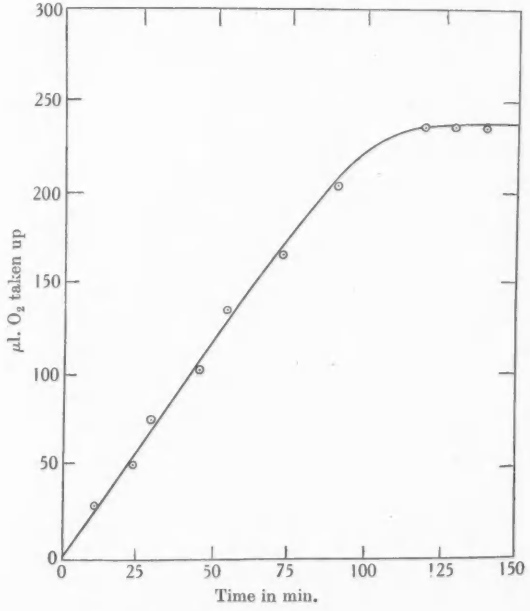


Fig. 5.

Sodium fluoride to a final concentration of 1/300 (0·2 ml. of *M*/20 solution) was added to each cup to ensure freedom from bacterial contamination. With 1 ml. of *M*/50 glutamic acid the oxygen consumed was 230 $\mu$l. This corresponds to 11·5 l. of oxygen per mol. or slightly over one atom of oxygen per molecule of glutamic acid.

Table VIII. *Oxygen consumption with 1 ml. of M/50 glutamic acid*

| Time in min. | Exp. | μl. oxygen taken up | |
|---|---|---|---|
| | | Control | Difference |
| 10 | 30·6 | 10·1 | 20·5 |
| 20 | 45·7 | 20·5 | 25·2 |
| 30 | 98·7 | 31·7 | 67·0 |
| 40 | 122·6 | 38·9 | 83·7 |
| 50 | 153·1 | 43·1 | 110·0 |
| 60 | 193·2 | 51·0 | 142·0 |
| 70 | 216·7 | 61·7 | 155·0 |
| 80 | 240·7 | 68·2 | 172·5 |
| 90 | 260·5 | 73·5 | 187·0 |
| 100 | 285·3 | 80·1 | 205·2 |
| 110 | 308·2 | 86·2 | 222·0 |
| 120 | 320·0 | 90·0 | 230·0 |
| 130 | 325·5 | 95·5 | 230·0 |
| 140 | 328·7 | 98·5 | 230·2 |

*Isolation of α-ketoglutaric acid as the end product of the oxidation*

To 250 ml. of the enzyme solution in *M*/15 phosphate buffer at *p*H 7·8, 100 ml. of *M*/10 glutamic acid neutralized with NaOH and 0·079 g. of arsenic trioxide as sodium arsenite were added and the mixture gently shaken for 2 hr. in a stoppered 500 ml. flask. It was found helpful to add 10 ml. of 1/1000 methylene blue to the mixture, the disappearance of the colour indicating the necessity of admitting fresh air and its permanence marking the end point of the reaction. At the end of 2 hr. the proteins were precipitated by adding 50 ml. of 50% trichloroacetic acid, the liquid filtered at the pump using filter paper impregnated with kieselguhr and the filtrate concentrated to 150 ml. *in vacuo*. 100 ml. of a 2% solution of 2:4-dinitrophenylhydrazine in 2*N* HCl were now added and the mixture kept in the refrigerator for 12 hr. The crystalline yellow precipitate which formed was filtered and purified by dissolving in 2*N* NaOH and reprecipitating with HCl. The precipitate thus obtained was separated on the centrifuge, washed several times with water and recrystallized from ethyl acetate. Golden yellow needle-shaped crystals of the dinitrophenylhydrazone of α-ketoglutaric acid melting at 223° were obtained. Yield 700 mg.

In a control experiment without the addition of glutamic acid 11 mg. of the same product were obtained. It is assumed that this arose probably from the glutamic acid formed in the breakdown of protein which was not completely removed by the method adopted for the purification of the enzyme.

## Summary

A number of germinating seeds have been examined for the presence of amino-acid dehydrogenases. The only such dehydrogenase found is one specific for glutamic acid which occurs in the seedlings of *Phaseolus mungo*, *P. radiatus* and *Pisum sativum*.

The enzyme is readily obtained in aqueous extracts free from cellular material and can be concentrated by precipitation with ammonium sulphate and redissolving in phosphate buffer. In this condition it actively oxidizes the naturally occurring *l*(+)glutamic acid, differing in this respect from deaminases from animal sources hitherto studied, which in cell-free extracts act only on the optically non-natural isomerides of the amino-acids.

The enzyme belongs to the class of aerobic dehydrogenases, being capable of oxidizing glutamic acid either in the presence of methylene blue or of molecular

oxygen. Hydrogen peroxide formation has been demonstrated by the coupled oxidation of nitrite. Cyanide, arsenite and fluoride do not inhibit the enzyme nor is it activated by methylene blue, ascorbic acid, glutathione or dihydroxyphenylalanine.

The oxygen uptake corresponds to 1 atom of oxygen per molecule of glutamic acid. The end product of the oxidation is α-ketoglutaric acid, which has been isolated and identified as the 2:4-dinitrophenylhydrazone.

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# CXLII. A SIMPLE GLASS ELECTRODE OF SMALL CAPACITY

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Numerous glass electrodes in which the electrode is a relatively long thin-walled tube have been described in the literature [McInnes & Belcher, 1933; Michaelis, 1936; Pickford, 1937; Dill *et al.* 1937], but all demand for their manufacture considerable skill in glass-blowing. The following is a description of one which has a capacity of 0·2 ml. or less, which requires no special skill in manufacture, and which can be used for measurements at temperatures higher than room temperature. In conjunction with a McFarlane-Pye valve potentiometer, it has been found to give excellent results on a variety of biological fluids, including blood.

The membrane itself consists of a capillary of Corning No. 015 glass about 6–9 in. long and bent to the shape shown in Fig. 1. After bending, the electrode is mounted in a split rubber cork of suitable dimensions to fit the mouth of a cylindrical histological staining tube. This is best done by cutting two small grooves in one or both halves of the cork to allow the capillary to be held without distorting the shape of the cork. A suitably sized hole should be bored in each half of the cork, one to admit the tip of a calomel half-cell and the other to carry a small thermometer. When the capillary is placed in position between the two halves of the cork a wire band around the cork will hold it securely and facilitate subsequent handling.

After mounting in its cork, the capillary is filled with some conducting solution, e.g. a buffer, and the whole assembly inserted into a staining tube previously waxed on its internal surface and filled with hydrofluoric acid. A fine platinum wire is inserted into the interior of the straight limb of the electrode and a second platinum wire dips into the surrounding acid. These wires are connected through a galvanometer and 10 megohm grid leak resistance to a 60 V. battery (Fig. 1). The grid leak acts as a protection to the galvanometer in case of a sudden puncture of the electrode.

On first setting up, the galvanometer reading indicates a resistance across the glass membrane of some 300 megohms, but as etching proceeds the galvanometer reading rises as the resistance falls and gives an excellent indication of how the etching is progressing. It has been found advisable to remove the electrode every minute or so and wash under a gentle stream of water. This renders the etching more uniform, presumably by removing adherent particles of fluorides. With care an electrode may be brought to as low a resistance as 10–20 megohms without puncturing, but, while it is desirable to get the resistance as low as possible, satisfactory results have been obtained with electrodes of resistances up to 50 megohms.

After etching, the electrode is removed from the hydrofluoric acid and immersed for at least 24 hr. in distilled water. The water may then be replaced by a solution of $N/10$ KCl in a phosphate buffer of $p$H 6·8.[1] This fluid is identical

[1] 0·47 g. $Na_2HPO_4$ + 0·45 g. $KH_2PO_4$ to 100 ml. $N/10$ KCl.

with that used in one of the half-cells; consequently when the electrode system is fitted up, as in Fig. 2A, there is no contact potential between this half-cell and

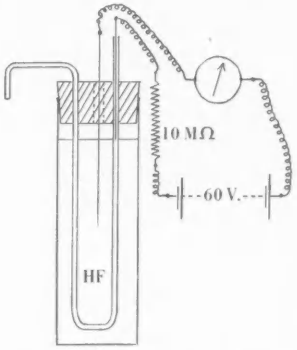


Fig. 1. Etching apparatus.

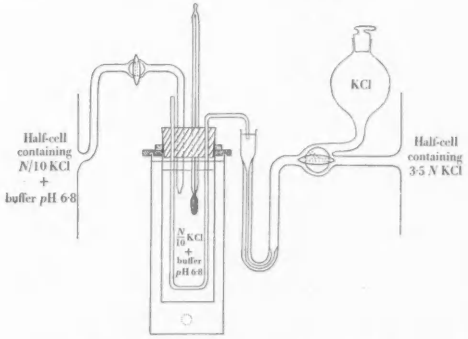


Fig. 2A. Side-view of electrode system with heated water-jacket.

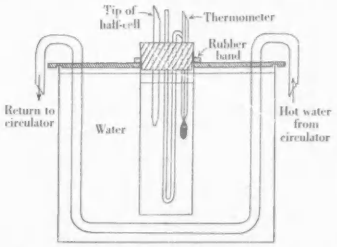


Fig. 2B. End-view of electrode system with heated water-jacket (half-cells omitted).

the fluid on the outer side of the glass membrane. A thermometer is inserted in one of the holes in the rubber cork and the electrode is ready for use.

In use, the capillary is filled simply by dipping the bent end into the fluid whose *p*H is to be determined, when the fluid will run in by capillary attraction.

The electrode assembly is connected to the half-cells as shown in Fig. 2 A. As previously stated, the tip of one of these half-cells dips into the fluid surrounding the capillary. The other half-cell, which contains 3·5 *N* KCl, ends in a U-shaped tube of narrow bore whose open end widens into a cup. The bent end of the capillary is slightly immersed below the surface of the KCl in this cup and the only liquid junction is at this point. In spite of its apparently crude nature, potentials stable over 2–3 hr. to within 0·2 mV. are obtainable in determinations on buffer solutions.

When measurements at body temperature are desired, the electrode is supported by a rubber ring in a rectangular specimen jar of suitable size filled with water. A glass tube passes down each end of this jar and along its bottom. The external ends of this tube are connected by rubber tubing to a copper spiral immersed in a heated water bath situated well below the electrode table. Convection maintains a circulation of hot water and, by manual adjustment of the flame below the water bath, it is possible to maintain the temperature around the electrode with sufficient accuracy for most purposes. A thermo-regulating system can be fitted if desired. Electrical heating of the water around the electrode was tried but had to be abandoned owing to interference with the potentiometer.

Used in conjunction with a McFarlane-Pye potentiometer, the following results were obtained on a series of twelve buffer solutions, ranging with varying intervals between *p*H 3·88 (No. 1) and *p*H 9·19 (No. 12). The potential differences between each pair of adjacent buffers was measured once by hydrogen electrode, and once by each of three glass electrodes of the above pattern. All measurements were made at, or corrected to, 20·5°.

| Buffer no. | $\Delta E$ by hydrogen electrode mV. | $\Delta E$ by glass electrode I mV. | $\Delta E$ by glass electrode II mV. | $\Delta E$ by glass electrode III mV. | Diff. $\Delta E$ hydrogen and mean $\Delta E$ all glass electrodes mV. | Diff. hydrogen and glass electrodes *p*H |
|---|---|---|---|---|---|---|
| 1 | | | | | | |
| 2 | 96·5 | 95·9 | 96·1 | 96·3 | −0·4 | −0·007 |
| 3 | 18·1 | 18·0 | 18·1 | 18·3 | 0 | 0 |
| 4 | 18·2 | 18·5 | 18·3 | 18·0 | +0·1 | +0·002 |
| 5 | 15·0 | 15·0 | 15·0 | 15·1 | 0 | 0 |
| 6 | 12·1 | 12·2 | 12·3 | 12·1 | +0·1 | +0·002 |
| 7 | 13·0 | 13·0 | 12·7 | 13·1 | −0·1 | −0·002 |
| 8 | 17·0 | 17·4 | 17·5 | 17·5 | +0·5 | +0·009 |
| 9 | 26·8 | 27·2 | 27·3 | 27·2 | +0·4 | +0·007 |
| 10 | 32·6 | 32·6 | 32·5 | 32·7 | 0 | 0 |
| 11 | 36·5 | 37·3 | 37·0 | 36·9 | +0·6 | +0·010 |
| 12 | 19·0 | 18·5 | 17·8 | 17·7 | −1·0 | −0·017 |


## Summary

A capillary glass electrode of extremely simple construction is described.


We wish to record our indebtedness to the Rankin Medical Research Fund for defraying the cost of the potentiometer.

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# CXLIII. A SPECTROGRAPHIC EXAMINATION OF THE MINERAL CONTENT OF HUMAN AND OTHER MILK

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The mineral content of milk has been the subject of many investigations by spectrographic and other methods, and the presence of certain elements has been definitely established. Regarding the less abundant constituents, however, there is still much uncertainty, the findings of different experimenters showing considerable disagreement. This is doubtless due in part to actual differences in the composition of the samples examined, but a review of the methods employed suggests that the technique has often been inadequate for definitive results with respect to substances present only in small traces. Further, most of the work has been done with cow's milk, the mineral content of human milk having been almost entirely neglected.

The present investigation has been undertaken with the object of obtaining information as complete and unambiguous as possible concerning the minerals present in various samples of milk commonly used in the feeding of infants. It was also considered desirable to ascertain whether manufactured milks, both dried and tinned, showed any significant difference in their mineral contents from fresh cow's milk. Quantitative results cannot yet be obtained with sufficient precision to justify publication, but a knowledge merely of the constant presence or absence of an element is of some importance in view of several known instances in which an element exercises a biological influence quite disproportionate to its abundance.

## *Preparation of the material*

Thirty samples of human milk and twenty-six of cow's milk in various forms were examined; details are given below.

(i) *Human milk.* The samples for the main investigation (modifications for subsidiary analyses will be described later) were extracted from 22 patients in normal health at the Royal Hospital, Wolverhampton, at various intervals from 3 to 17 days after confinement. Two quantities were taken at different times from each of 8 of the patients, bringing the total number of samples to 30. The milk was expressed from the breast direct into a clean test tube, the nipple being placed inside the tube so as to avoid contamination from the skin. The test tubes employed were new and were prepared by three preliminary boilings in dilute nitric acid, and washed out in glass-distilled water. The milk was then dried at 100° in a steam oven and the dried material sent to the Imperial College in small glass tubes which had been similarly cleaned. On arrival each was very gently charred in a clean porcelain crucible and returned to its original bottle in the form of a black powder.

(ii) *Other milk.* Three specimens of cow's milk were taken from the pail immediately after milking and treated in a similar manner to the human milk. The manufactured milks were taken from the containers and ashed on Vitreosil

capsules, cleaned by boiling in dilute HCl, the ash being sent to the Imperial College. The milks examined were as follows:

1. Nestlé's Condensed Milk. Fluid milk in a tin.
2. Nestlé's "Ideal" Milk. Fluid milk in a tin.
3. "Secway." Dry milk. Tin with paper wrapping.
4. Mead's "dextri-maltose". Powder in tin. No paper.
5. Allenbury's Humanized Milk Food. No. 1. Dry milk. Tin with paper.
6. Allenbury's Humanized Milk Food. No. 2. Dry milk. Tin with paper.
7. Allenbury's (Malted) Milk Food. No. 3. Dry milk. Tin with paper.
8. Humanized Trufood. Dry milk. Tin. No paper.
9. Ostermilk No. 2. Dry milk. Tin with paper wrapping.
10. Cow and Gate. (Full Cream.) Dry milk. Tin with paper wrapping.
11. Savory and Moore. Dry milk. Tin with paper.
12. Mellin's Food. Dry. Glass with brass top but no paper wrapping.
13. Horlick's Malted Milk. Dry milk. Glass with tin top but no paper.
14. Virol and Milk. Dry milk. Tin with paper wrapping.
15. Lactogen (Nestlé). Dry milk. Airtight tin with paper wrapping.
16. Lacidac (Full Cream) (Cow and Gate). Dry. Tin with paper wrapping.
17. Dorsella (English Full Cream). Dry milk. Cardboard. Paper wrapping.
18. Eledon (Nestlé). Dry milk. Tin with paper wrapping.
19. Benger's Food. Dry milk. Tin with paper wrapping.
20. Dorsella. Humanized Milk. Dry. Tin with paper wrapping.
21. Libby's Unsweetened Evaporated Milk. Tin.
22. Kingsway Condensed Milk. Sweetened. Fluid in tin.
23. Dairy-Man Pure Devon Cream. Fluid in tin.

### *Analysis*

The arc spectrum of each specimen was photographed. For this purpose a portion was placed in the first instance on graphite electrodes, and the arc was struck at 220 V. The spectrum of the electrodes alone was photographed previously by prolonged exposure in every case, and the spectrum of the milk was photographed in an adjacent position. A comparison spectrum of the iron arc was then photographed on the other side of the milk spectrum to enable the lines to be identified. All the specimens were treated in the same manner, the effective exposure time being about 1 min., during which three successive portions of the material were supplied to the arc at approximately equal intervals.

The graphite electrodes, though fairly pure, contained small proportions of a few impurities, the chief of which were Ti, V, B and Si. For the detection of such elements in the milk, fresh spectra were taken by an identical process, but with copper instead of graphite poles. This was necessary, since the lines of an impurity in the electrodes are often very greatly strengthened when metals are inserted into the arc, even though none of that impurity is added. The presence of an element in the milk was therefore not considered established unless it revealed itself on electrodes which themselves showed no trace of the element. This point will be further discussed later.

Two spectrographs were used for the greater part of the investigation. The region λλ6720–3700 was examined with a glass instrument having separate collimator and camera tubes and giving a dispersion ranging from 25 Å./mm. in the red to 3·7 Å./mm. in the violet; and the ultra-violet region, from about λλ3560 to 2530, with a quartz Littrow spectrograph (Hilger Type E 1), with dispersion ranging from 6·7 to 2·8 Å./mm. These instruments were so disposed as to photograph the spectrum of a single source simultaneously, the source

being situated between the two slits on the common line of collimation. In spite of the large dispersion, therefore, the whole region from λλ6720 to 2530 (with the exception of the small gap between λλ3700 and 3560, which is unimportant for the purpose in view) was photographed at once. Almost all elements detectable by spectrographic analysis have sensitive lines falling within this range; the only exceptions are B—best indicated by a close pair of lines at λλ2498, 2497—and Rb which, though it gives a sensitive pair at λλ4216, 4202, is best detected by the still more sensitive pair in the infrared at λλ7948, 7800, since the violet lines fall in a strong part of a cyanogen band which is almost certain to appear in a graphite arc in air. For these elements, therefore, separate exposures were made on other instruments suitable for the purpose.

The spectra obtained had considerably larger dispersion than has been usual in this work, but not larger than is necessary for certainty with respect to several of the elements concerned. The only comparable dispersion appears to be that employed by Blumberg & Rask [1933] who used a Hilger E 1 spectrograph throughout, and thus obtained similar ultraviolet spectra but a considerably smaller dispersion in the visible region. Blumberg & Rask's investigation, however, was limited to cow's milk, and since only graphite electrodes were used, the results were indefinite with respect to the elements occurring therein as impurities.

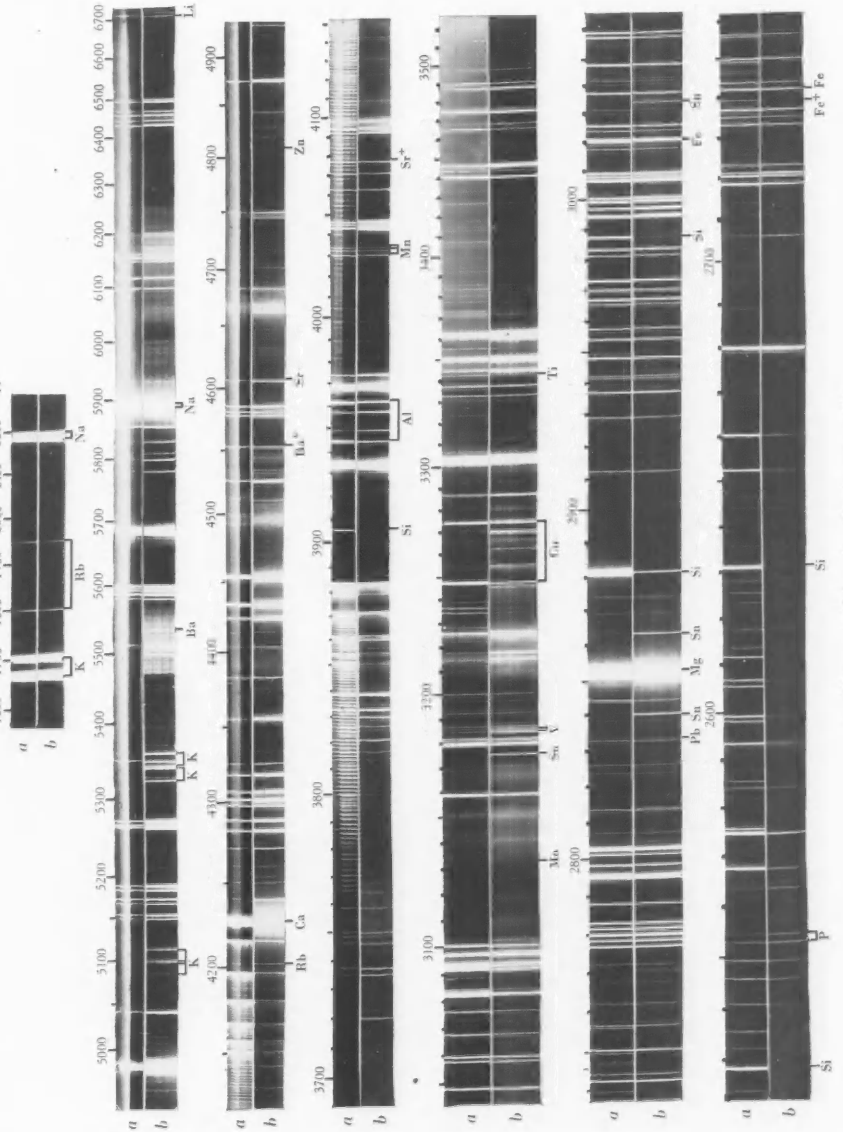
*Preliminary indications*

The spectra were examined for the presence of 51 elements. Each specimen of non-human milk was examined separately for each element, and for most of the elements each specimen of human milk also was so examined. On account of reduction of the material, however, the additional plates on copper poles for the detection in human milk of Ti, V, B and Si, and those in the infrared region for the detection in human milk of Rb, were obtained with a mixture of all the 30 specimens. The almost identical character in most other respects of the various samples of human milk makes it unlikely that there was any marked variation in the content of these elements.

In Plate IX are shown the spectra of two mixtures—one of all the human and the other of all the non-human specimens—taken under the same conditions on carbon electrodes (which, for a reason which will be appreciated later, were chosen on account of their small content of Ti and V, although in other respects they were less pure than the graphite poles used in the actual analysis) which showed identical spectra by themselves. Pronounced differences between the spectra on the plate may therefore with some confidence be attributed to variations in the respective mixtures.

It would be illegitimate, however, to regard the strength of a line as giving a direct indication of the abundance of the corresponding element, chiefly for the following reason. A certain proportion of the atoms of most elements become "ionized" in the arc (i.e. the atom is deprived of an electron), and an ionized atom gives a different spectrum from an ordinary neutral atom. Consequently, the more an element is ionized the weaker will the lines of its neutral atom appear. Now the spectra show that the degree of ionization is greater for the human than for the non-human milk.[1] This may be seen by comparing lines of neutral and ionized atoms, respectively, of a single element. Fe provides a striking example: the line of neutral Fe at λ2742 is only slightly weaker in the spectrum of non-human milk, while the neighbouring line (marked $Fe^+$) of ionized Fe at λ2739

[1] This is doubtless due to the obvious excess of K in the non-human milk. K is an element which is very easily ionized, and the electrons which are set free by its ionization tend to recombine with ionized atoms of other elements and increase the proportion of neutral atoms of those elements.




*a* = Human milk. *b* = Non-human milk.

is scarcely visible in that spectrum. A similar effect is shown by other elements—e.g. Ba and Sr (compare $\lambda$5536 of Ba with $\lambda$4554 of $Ba^+$, and $\lambda$4607 of Sr with $\lambda$4077 of $Sr^+$). It follows that if we are to get a true estimate of the relative abundance of the elements from the strength of the spectrum lines, we must suppose the neutral lines (including all marked lines which have no + sign attached to the chemical symbol) of each element in the spectrum of human milk to be somewhat strengthened.

Absolute results cannot, of course, even then be obtained, owing to unavoidable differences in time of exposure, quantity of material used etc. We may, however, choose an element as standard and obtain results relative to that. P is suitable for this purpose, since its lines (at $\lambda\lambda$2555, 2553) are approximately equal in strength in the two spectra when the spectrum of human milk is suitably augmented as already suggested. The only striking differences which then appear are an excess of K and Sn in the spectrum of non-human milk, and of Si, Cu and Fe in that of human milk. Of these, the Sn may be attributed to the containers, and it is doubtful if the excess of Cu and Fe in human milk has much importance since each of these elements showed wide variations from one specimen to another of each kind of milk. Their occurrence is discussed in more detail below. The results for K and Si, however, appeared to be significant. Each of these elements also varied in abundance in the individual specimens, but on the whole their excess in their respective kinds of milk seemed at first to be quite definite.

It was noticed, however, that none of the three specimens of fresh cow's milk showed Si, and this fact suggested that possibly the apparent excess of this element in human milk was due to solution from the glass in which it was collected. Examination of further specimens collected in Si-free vessels confirmed this suggestion, and in order to avoid false conclusions with regard to other elements, a subsidiary series of experiments was made.

*Subsidiary experiments.* For these experiments fresh samples of human milk were obtained in various containers, the object being to obtain evidence of the presence of as many elements as possible under conditions which precluded the possibility of such elements having been introduced outside the human body. Since human milk, in the normal process of breast feeding, passes direct from the mother to the child, it is clearly important to take into account every possible source of contamination.

It may be presumed that solution from the atmosphere is negligible. The only elements detectable spectroscopically which, so far as is known, could have been introduced in this way, are Na, C and Hg: the first two are known to be present in milk, and the last was not detected in any of the spectra. Care was, of course, taken to avoid dust. The remaining possible sources of impurity are three in number, viz. the vessels in which the milk was collected, the crucibles used for the preliminary charring, and the electrodes used for the arc.

Specimens were obtained, by the process already described, in vessels of copper, rubber, zinc, and platinum. They were charred in crucibles of porcelain, glass, copper, zinc and platinum, and examined with electrodes of graphite, copper and zinc. At least three specimens were obtained in each kind of vessel (except platinum, only one tube of this material being available), and all the vessels, crucibles and electrodes were subjected to separate spectroscopic investigation to ascertain their composition. In order to ensure that these subsidiary specimens were in no way abnormal, milk from the same woman was collected in every case, as nearly as possible at the same time, in glass tubes and treated exactly as in the main investigation. Comparison with the earlier spectra showed that the new material was identical with the old. It should be

added that the collecting vessels, crucibles and electrodes used in the main investigation were also separately analysed.

It was hoped in this way to determine definitely whether each element revealed in the main experiment was actually contained in human milk or introduced after extraction. The evidence obtained appears to be conclusive except for four elements, B, Cu, Fe and Pb, for which, for reasons stated below, the results are less certain than those for the other elements.

## Results

In estimating the mineral content of milk, two points need consideration: (1) the universality, (2) the certainty, of the presence of each element. The main investigation gave evidence on the former, and the subsidiary experiments on the latter of these points. In the following statement, the various elements concerned are dealt with separately in the first instance, and the conclusions are afterwards summarized.

(1) In all the specimens examined, of both human and non-human milk, the following elements were detected, and confirmed by the subsidiary experiments as constituents of human milk: Ca, Mg, Na, P, Ba, Mn. Rb may safely be included among these elements, for it appeared in every specimen of non-human milk and in the mixture of all human milks. As already stated, it was not looked for in the separate samples of the latter.

It is surprising to find Ba in this list. It was not found by Wright & Papish [1929], or in the Indian milks examined by De [1935], but was present in all of 19 samples of cow's milk examined by Blumberg & Rask [1933]. Its constant presence in the present series suggests that it may be an essential element in nutrition. There are certain observations in support of this, for Ramage & Sheldon [1931] found it regularly in the choroids of cattle in amounts increasing with age, though it was not found in other species. Gerlach & Muller [1936], using a different spectrographic technique, found it in the choroid of every specimen of all the species examined, and also found that the greatest amounts were present in cattle. Rb is found so constantly in tissues that its constant presence in milk is not surprising, and the observation is in accord with that of Ramage *et al.* [1933] that the amount of Rb in the infant's liver appears to rise slightly during the nursing period.

(2) The following elements were found and confirmed in almost every specimen: K, Sr, Li, Zn, Al. The character of the exceptions, indicated in the following notes, suggests that these elements may be included with those mentioned in the previous paragraph as normal constituents of milk.

(*a*) K was found in all non-human milks and in all except two specimens of human milk. These two came from the same woman, 3 and 8 days after confinement, respectively. Both spectra were generally weak, but when, by prolonged exposure and use of additional material, they were brought up to average strength, there was still no certain evidence of K. If the extreme red region had been examined the element would possibly have shown itself, but there is no doubt that the milk of this woman was abnormally deficient in K. On the whole, K was decidedly stronger in non-human than in human milk.

(*b*) Sr was found in all non-human and in all but one specimen of human milk. A sample taken from the same woman 3 days later, however, showed this element.

(*c*) Li was never strong enough to reveal its presence by more than one line—at $\lambda 6708$ in the red. This is not an easy region to observe, and in a few specimens

of both types of milk the line could not be detected with certainty. The most probable interpretation of the results is that Li is normally present in milk in very small quantity.

(*d*) Zn was found in very small traces in all specimens except two of non-human and three of human milk. Each of the three human specimens, however, came from a woman in earlier samples of whose milk Zn was found. This suggests that the Zn content of human milk might decrease as lactation proceeds, a possibility which is supported by the fact that the specimens in which this element was most pronounced were obtained during the first few days. This observation is in complete agreement with that of Sato & Murata [1932]. Using a chemical method they found that human, cow's and ewe's milks showed high figures for Zn immediately after parturition, with a marked fall to a steady level after 10–15 days.

(*e*) Al was very weak in all specimens except one of non-human and two of human milk, which failed to show the element at all; it appeared, however, in a later sample from the same source as one of the exceptional human specimens.

(3) Cu appeared in every spectrum examined, but we did not succeed in obtaining electrodes spectroscopically free from this element. It was present also in slight traces in all collecting vessels, and the greatly increased strength of its lines in the spectra of milk collected in copper tubes shows that milk is able to dissolve copper in (spectrographically) large amounts. This element must therefore be considered as possibly present in small quantity in milk.

(4) Fe is subject to uncertainty for the same reason as Cu. The most Fe-free material obtained was zinc, which showed the presence of Fe by only one line, at $\lambda 2483$, and in the specimens collected, treated and examined in zinc this line showed no clear enhancement in strength, and no other Fe lines appeared. It may be said, therefore, that there is no unquestionable spectrographic evidence of the presence of Fe in milk. In the spectra obtained in the main investigation, however, Fe showed greater variation than most elements, though all specimens were treated similarly. It is possible, therefore, that Fe is an irregular constituent of milk.

Davidson & Leitch [1934] report a conflict of chemical evidence concerning the amount of Fe in milk, though there appears to be general agreement that it is present. The estimates range from about 0·17 to 0·02 mg. per 100 ml. for human milk, and the variation is somewhat greater for cow's milk. Considerable changes during lactation are reported. This is not qualitatively inconsistent with our results. We have made experiments to determine the effect on the spectrum of adding known small quantities of Fe to specimens of milk, and find that, while the addition, to amounts equal to those used in the investigation, of Fe in the proportion 0·02 mg. to 100 ml. produces a definite strengthening, the change is less than the variation from one unadulterated specimen to another.

While the evidence is inconclusive, therefore, its general tendency is towards a low, and possibly a zero, Fe content for human milk. The results indicate that it would be worth while to look for chemical evidence of Fe in samples of milk collected in and treated with materials in which Fe is not detectable chemically. Owing to our failure to obtain electrodes spectroscopically free from Fe, we cannot say with certainty whether or not glass is such a material. Experiments we have made, however, strongly suggest that glass taken from test tubes is not free from Fe, while fused silica probably is.

(5) B and Si appeared in most specimens of non-human milk and in the mixture of all human milks in the main investigation. As already remarked, however, Si did not appear in the spectra of human milk in the subsidiary

experiments in which it could not have appeared as an impurity. There is thus no evidence of the presence of Si in milk.

It was thought that B might also have been introduced from the glass containers, since the spectrum lines of this element are well known to occur as impurity lines in vacuum tube spectra, but the evidence on this point is not conclusive. In the main investigation B was absent from only two specimens of non-human milk, and it appeared in the spectrum of the mixture of human milks, but in no instance was more than a slight trace detectable. It appeared in fresh cow's milk, which showed no Si, and there appeared in general to be no relation between the strengths of the B and Si lines in the various spectra. In the subsidiary experiments, B was found in none of the spectra where its appearance would have been conclusive, but in view of the lower sensitivity of the test with copper and zinc electrodes, and the fact that under the best conditions it was only just detectable, it cannot be said with confidence to be absent. The irregular strength of the B lines in the various spectra suggests that this element is sporadically present in minute traces in milk.

(6) In the main investigation Pb appeared faintly in all but five specimens of human milk, but in only eight samples of non-human milk, all of which had been commercially prepared. Seven of these samples of non-human milk showed Sn also; the other (a dry milk packed in glass with brass top) showed Pb as strongly as any sample, but no trace of Sn. It would be unsafe, therefore, to conclude that the Pb in non-human milk came from an impurity in the containers though it may, of course, have been introduced in the process of preparation.

No container used for human milk was found spectrographically free from Pb, though in some it was only just detectable. It appears unlikely that such minute amounts would be dissolved selectively in sufficient quantity to be observed, and the non-appearance of Pb in the spectra of some specimens collected in vessels containing this element supports the conclusion that Pb is a possible constituent of milk. If this be accepted, the evidence suggests that the Pb, like the Zn, content of human milk tends to decrease with time after confinement. Of the five Pb-free samples of human milk in the main investigation, three came from women in earlier samples of whose milk the element was found. It appears also that Pb occurs more consistently in human than in generally available non-human milk.

(7) Mo was very weakly represented in all but five samples of non-human milk, but in none of the human milks examined separately, though it was just detectable in the mixture of all of them. Its relative strength in non-human milk may, of course, be attributable to acquisition of this element during the process of preparation.

(8) A trace of Sn was found in only one specimen of human milk, extracted 8 days after confinement. A sample extracted from the same woman 12 days after confinement did not show this element: its presence in the former sample may therefore probably be considered accidental. The non-human milks showed great variations in the abundance of Sn, doubtless due to the manner of packing. As would be expected, milks packed in contact with the container showed Sn lines most strongly, fluid milks being decidedly richer in Sn than the dry samples. None of the specimens of fresh cow's milk showed this element.

The final conclusions reached may be summarized as follows:

(I) The following elements, roughly in order of spectrographic prominence, may be regarded as normal constituents of both human milk and the commercial milk foods examined: Ca, Mg, Na, P, K, Rb, Li, Ba, Sr, Mn, Al, Zn.

(II) Pb and B are probably normal constituents of both kinds of milk, which occur in minute and irregular quantity.

(III) The presence of Cu and Fe in milk is doubtful.

(IV) Mo in minute traces is possibly a normal constituent of milk.

(V) No evidence has been obtained of any of the following elements in pure milk: Sb, As, Be, Bi, Cd, Cs, Cr, Co, F, Ga, Ge, Au, In, Ir, La, Hg, Ni, Nb, Os, Pd, Pt, Rh, Ru, Sc, Si, Ag, Ta, Tl, Sn, Ti, W, V, Y, Zr.

The complete absence of Co is somewhat unexpected, for it is an element with active biological properties. Not only is it capable of causing polycythaemia, but recent Australasian work [cf. Askew & Dixon, 1936] suggests that, at any rate in the sheep, it is a necessary element in nutrition.

(VI) The human and non-human milks are in most respects very similar so far as mineral content is concerned; the differences between them are, in general, smaller than the variations between different specimens of either kind. On the whole the human milk specimens resemble one another rather more closely than the others, as might have been expected. There may, of course, be quantitative differences, of which only the roughest indication can be obtained from these observations; nevertheless, one or two general conclusions can be drawn. The most conspicuous difference is an excess of K in non-human milk, but there is no evidence as to whether this is introduced in the process of preparation. Smaller differences are a relative excess of Mo and defect of Pb in non-human milk, in the state in which it was examined. From the practical point of view, however, it is immaterial how the elements present are acquired, and it must therefore be recorded that the baby fed on commercially prepared non-human milk receives Sn and Si in varying amount, neither of which elements is absorbed by the breast-fed baby.

(VII) Fresh and commercially prepared cow's milks are very similar in mineral content. The only noticeable differences are that none of the three specimens of fresh cow's milk showed evidence of Si, Sn or Pb, which elements were irregularly present in many of the 23 specimens of prepared milk, and that in every specimen of fresh cow's milk Fe was much weaker than in the average of the prepared milks. Si and Sn are undoubtedly, and Pb and Fe possibly, introduced into the prepared milk after extraction from the cow.

*Comparison with previous observations*

No useful purpose would be served by comparing these results in detail with those obtained by other investigators because the samples examined and the conditions of experiment vary so widely. The only other spectrographic analysis of human milk that we have found was made by De [1935], who compared the spectra of five samples of milk from Indian women with an equal number of samples of each of cows', goats' and buffaloes' milk, respectively. De gives a table comparing the results of five analyses, by separate investigators, of various kinds of milk, and the disagreement in the results is indicated by the fact that, out of 21 elements included, only 8 were found by all five. These were Ca, Mg, P, Na, K, Cu, Ti and Zn. B and V may perhaps be added, since these elements were found in four cases and were not tested for in the fifth.

The most striking divergence of the present results from these is concerned with the elements Ti and V. No evidence has been found that these elements occur in milk, though their lines appeared strongly on all spectra taken with graphite poles, even when the poles themselves, by prolonged running of the arc, had been so cleared of impurities at their extremities that no metallic lines appeared in their spectra. That this treatment is insufficient to permit safe

conclusions to be drawn with regard to Ti and V was proved by adding to poles so treated a little "specpure" $CaCl_2$ known to contain neither of these elements; Ti and V lines nevertheless appeared in strength almost incredible without actual experience of the phenomenon. In Plate IX Ti and V are well represented, although in the spectra of the carbons alone they were barely detectable. In the light of these experiments it appears invalid to deduce the presence of Ti and V from the occurrence of their lines in spectra taken with graphite poles, which have generally been used for this work. No sign of these elements appeared in spectra taken with copper poles. The copper arc is admittedly less sensitive than the graphite arc in the detection of small traces, yet it showed Sr and Ba quite definitely, although those substances were much less prominent than Ti and V in the graphite spectra. We are forced to the conclusion that if Ti and V occur in milk of any of the kinds examined, their abundance is too small for ordinary spectrographic detection.[1]

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[1] Webb & Fearon [1937] have called attention to this effect, and they include Mo among the elements which should be suspected on this account. We have, however, found no trace of Mo in the graphite used in this investigation, and the marked difference between human and non-human milks with respect to this element makes it very unlikely that the Mo lines observed arise from the electrodes.

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# CXLIV. DEPHOSPHORYLATION IN MUSCLE EXTRACTS

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The dephosphorylating action of muscle extract on various substrates all of which are not necessarily intermediate products formed during normal glycolysis is very well known. The presence of the phosphatases, inorganic pyrophosphatase, adenylpyrophosphatase and hexosediphosphatase has been demonstrated [Lohmann, 1928, 2; 1933] and the enzymes catalysing the transfer of phosphate from creatinephosphate and phosphopyruvic acid to adenylic acid have been studied in greater detail. The dephosphorylation of phosphopyruvic acid like that of creatinephosphate has hitherto been considered to involve only a straightforward transfer of the phosphate to adenylic acid. This reaction has now been investigated further and it will be made clear that it is not by any means such a simple exchange as has been supposed.

In normal glycolysis where glycogen is the substrate it is easy to conceive of the adenylic acid being continuously esterified and reformed, the former during the dephosphorylation of phosphopyruvic acid, the latter by donating the phosphate to glycogen or hexosemonophosphate. This latter reaction will go on as long as any carbohydrate is left and will thus be regenerating the adenylic acid to act as acceptor for the phosphate from phosphopyruvic acid. The enzyme adenylpyrophosphatase would also help in the dephosphorylation but on the other hand the coupled esterification of adenylic acid recently demonstrated [Needham & Pillai, 1937; Meyerhof *et al.* 1937] would tend to remove some of the adenylic acid. It was supposed however that when hexosediphosphate is the substrate the adenylic acid is regenerated solely by the activity of the adenylpyrophosphatase since the hexose is already phosphorylated and cannot act in this way, and no other intermediate compounds are known which could serve as phosphate acceptor. An extract which does not contain adenylpyrophosphatase should therefore be incapable of producing lactic acid from hexosediphosphate when adenylpyrophosphate and not adenylic acid has been added as activator. This however is by no means the case and it therefore remained to be seen how phosphopyruvic acid could be dephosphorylated under these conditions. The possibility of coenzyme I acting in a capacity similar to that of adenylic acid, where it is alternately phosphorylated and dephosphorylated must be borne in mind but the mechanism of this reaction is still quite obscure even if it takes place at all [Ohlmeyer & Ochoa, 1937].

A fairly stable powder can be prepared from a fresh muscle extract by precipitation with acetone and drying: this gives an active extract with water and it is found that of the two pyrophosphatases present in the fresh extract, the adenylpyrophosphatase, which is comparatively weak even in the fresh extract, has almost completely disappeared while the inorganic pyrophosphatase is still quite active. The third phosphatase, the hexosediphosphatase has not suffered any serious inactivation. While this extract is therefore unable to decompose adenylpyrophosphate it was still found to be able to dephosphorylate

phosphoglyceric acid. That some hitherto unnoticed mechanism of phosphoglycerate dephosphorylation other than transfer through adenylic acid is here concerned is shown in a striking way by the fact that this extract cannot dephosphorylate creatinephosphate in the presence of adenylpyrophosphate. Further, this dephosphorylation of phosphoglycerate is increased several times by arsenate but with this difference: the activation by arsenate can be inhibited by iodoacetate while the original dephosphorylation is not.

The whole question of the mechanisms concerned in formation of inorganic phosphate in muscle is of interest, because in normal muscle contracting anaerobically increase in free phosphate, as well as in lactic acid and creatine, is always observed. Here again, the formation of the free phosphate has usually been explained by the action of adenylpyrophosphatase alone.

The present work includes some preliminary experiments on the pyrophosphatases and on hexosediphosphatase, particularly on separation of the enzymes and comparison of their activities. The dephosphorylation of phosphoglycerate was then investigated, especially the activation of this reaction by arsenate and the inhibition of the activation by iodoacetate. The experiments will be described in this order and the results examined and discussed at the end.

## Experimental details

### *Enzyme preparation*

The preliminary work on the pyrophosphatases was done on fresh muscle extract prepared by extracting fresh rat or rabbit muscle with ice-cold water or 0·5 % KCl for $\frac{1}{2}$–1 hr., centrifuging and dialysing the extract for 3–5 hr. For all remaining experiments, except when specified otherwise, aqueous extract of dried acetone powder from rabbit muscle prepared according to the method described in a previous paper [Needham & Pillai, 1937] was used. The extract was dialysed for 3–88 hr. according to the type of experiment and the details are given with each experiment. As will be evident from the experimental part of the work the acetone powder does not contain any adenylpyrophosphatase and it was not therefore necessary to keep the extracts for 5 days before use to inactivate this enzyme.

### *Chemical preparations used*

Adenylpyrophosphate was made according to the method of Lohmann [1931]. For cozymase the method described by Green *et al.* [1937] was used. Phosphoglyceric acid was made by the method of Vercellone & Neuberg [1935], barium creatinephosphate according to Meyerhof *et al.* [1937]. Adenylic acid was obtained from the Laakoon Factory, Lwow, Poland.

### *Methods of estimation*

Inorganic phosphate was estimated by the method of Fiske & Subbarow [1925]; when arsenate was present the Pett [1933] modification was used. The adenylpyrophosphate was estimated by means of its easily hydrolysable phosphate formed by 7 min. hydrolysis in *N* HCl at 100° [Lohmann, 1928, 1] after being separated from other interfering substances when present by barium precipitation. Creatinephosphate P was separated by barium precipitation of other phosphates and estimated according to Fiske & Subbarow [1929] when necessary; phosphopyruvic P was found by estimating P decomposed in 1 hr. hydrolysis in *N* HCl at 100°, with proper controls for small amounts of

other easily hydrolysable phosphorus compounds which might be present; the difficultly hydrolysable phosphate was found by subtracting the inorganic P present after 180 min. hydrolysis in *N* HCl at 100° from the total P. Pyruvate was determined by bisulphite treatment and subsequent iodine titration [Clift & Cook, 1932], lactic acid by the method of Friedemann & Graeser [1933].

*Experimental procedure*

For most of the preliminary experiments with pyrophosphatases 1 ml. of the fresh extract made up to 3–6 ml. with additions was used. 1 ml. of adenylpyrophosphate containing 0·55 mg. pyro-P, 1 ml. of 2·5 % $Na_4P_2O_7$, $10H_2O$ and 1 ml. of 1·5 % $MgSO_4$, $7H_2O$ were added to each when necessary, with sodium bicarbonate as buffer. The time of experiment was usually 1 hr. at 38°.

When the acetone powder extract was used for experiments with hexosediphosphate and phosphoglycerate 2 ml. extract were usually made up to 4–5 ml. with additions. The concentration of sodium hexosediphosphate was about 0·011–0·0125 *M*, of sodium phosphoglycerate 0·025 *M*, of A.T.P. 0·0006–0·0003 *M*, of cozymase 0·0003–0·0001 *M*. In the early experiments the arsenate concentration was 0·0025 *M*, later this was reduced to 0·001 *M*. The strength of iodoacetate used was in some cases 0·004 *M*, in other cases it was reduced to 0·0012 *M*. Sodium bicarbonate was used as buffer to give a final concentration of 0·45–0·22 %; the strengths of other additions are given in the proper places.

In all cases suitable controls were carried out simultaneously without addition of substrates but with all coenzymes and also with the substrates but without coenzymes.

Most of the experiments were done at 38° and the duration was 30 min.; those with arsenate were usually at room temperature (20°). Trichloroacetic acid was used to precipitate proteins and after filtration through paper P estimations were done on the filtrate. In the case of creatinephosphate P centrifuging was used in preference to filtration.

The results are expressed unless otherwise stated as mg. increase or decrease per ml. of extract.

*Pyrophosphatases in fresh muscle extract and in acetone powder extract*

Fresh muscle extract is known to contain a hexosediphosphatase, an inorganic pyrophosphatase and adenylpyrophosphatase [Lohmann, 1933]. Glycerophosphatase has generally been found to be absent. Of these the inorganic pyrophosphatase and the hexosediphosphatase are both quite stable and very active while the adenylpyrophosphatase is easily inactivated and is in any case not so active even in a freshly prepared extract as the inorganic pyrophosphatase. There is conflicting evidence in the literature as to whether these two pyrophosphatases are distinct and separate enzymes. Jacobsen [1931, 1933] claimed to have separated from liver an enzyme which was active towards adenylpyrophosphate but not inorganic pyrophosphate by selective destruction with 10 % $NH_4Cl$ at 55° but doubts on the validity of this have been thrown by other workers [Lohmann, 1933; Satoh, 1935; Haase, 1936]. It is a comparatively easy matter to get the inorganic pyrophosphatase free from the adenylpyrophosphatase as the latter is very easily inactivated. But it must be remarked in passing that the enzyme is not completely destroyed by keeping for 5 or 6 days in the ice-chest or by long dialysis. Certain extracts were found to have considerable adenylpyrophosphatase activity even after 2 weeks and dialysis for 16 hr. The

activity seems to vary a good deal with different extracts and a minimum of 3 weeks would seem to be necessary. Table I shows the adenylpyrophosphatase activity of an extract during the course of 2 weeks.

Table I

| | mg. P produced |
|---|---|
| Extract 5 days old, dialysed 8½ hr. | 0·42 |
| Extract 9 days old, dialysed 8½ hr. | 0·22 |
| Extract 11 days old, dialysed 16 hr. | 0·11 |
| Extract 13 days old, dialysed 8½ hr. | 0·12 |

0·55 mg. P present as pyrophosphate fraction of A.T.P.; time 1 hr. at 38°.

The fact that adenylpyrophosphatase so readily decreases in activity on dialysis or standing suggests the possibility that a coenzyme may be necessary.

Experiments were tried to see whether a solution freed from adenylpyrophosphatase by long standing could be reactivated by adding a fresh boiled extract which contained active adenylpyrophosphatase but no reactivation could be obtained. Table II shows the result of an experiment in which a fresh extract containing adenylpyrophosphatase acted upon inorganic and organic pyrophosphate separately and together and would seem to show that the enzymes were separate as the amounts of inorganic P produced are additive.

Table II

| | Inorganic P | | |
|---|---|---|---|
| | I | II | III |
| A.T.P. only | 0·35 | 0·38 | 0·28 |
| Na pyrophosphate only | 1·97 | 2·09 | 2·10 |
| A.T.P. + $Na_4P_2O_7$ | 2·58 | 2·87 | 2·57 |

The reason for the total being rather more than the sum of the individual values is not clear but the fact that the two dephosphorylations do not inhibit each other is evident.

Attempts were now made to separate the two enzymes by adsorption and precipitation methods. Three different aluminium hydroxides, kaolin and magnesium carbonate at different ranges of acidity and alkalinity were tried without success.

Finally it was found that the acetone powder muscle extract prepared by precipitating a fresh muscle extract with acetone and extracting the dried powder was quite free from adenylpyrophosphatase even immediately after preparation while it still contained both inorganic pyrophosphatase and hexosediphosphatase in large amounts. Most of the experiments with hexosediphosphate and phosphoglycerate described in the succeeding sections were done with such an extract.

The activity of such an extract towards both pyrophosphates will be seen from the following figures.

| | mg. P | |
|---|---|---|
| Adenylpyrophosphate | 0·02 | 30 min. at 38° |
| Sodium pyrophosphate | 0·72 | |

If inorganic pyrophosphatase is really a separate enzyme (as the evidence suggests) its function in the muscle is a matter for conjecture, for, so far as is known, inorganic pyrophosphate does not occur there.

As regards the glycolytic power of these extracts it may be noted that they are more active towards hexosediphosphate than glycogen but even with

hexosediphosphate their activity is rather low (1 mg. lactic acid per ml. extract from hexosediphosphate). This activity is not increased appreciably by arsenate; a fact to be contrasted with the great effect of arsenate on the dephosphorylation of phosphoglyceric acid to be discussed later.

*Dephosphorylation of sodium hexosediphosphate*

The dephosphorylation of hexosediphosphate in muscle extract has been investigated very fully by Lohmann [1933]. One might add that manganese, as might be anticipated, can replace magnesium as the activator as well or even perhaps better and in much smaller molecular concentration. Neither adenylpyrophosphate nor cozymase is necessary.

| | Inorganic P produced |
|---|---|
| Manganese (0·09 mg.) | 0·63 |
| Magnesium (0·15 mg.) | 0·57 |

It must also be noted that in an acetone extract some of the hexosediphosphate when present in the usual concentrations ($M/80$) might always, even in the presence of all the coenzymes (magnesium, A.T.P. and coenzyme I), break down into inorganic P and hexosemonophosphate although part is at the same time undergoing dismutation. A comparison of the dephosphorylations of both hexosediphosphate and phosphoglycerate when present in equivalent amounts as regards P (approx. $M/40$) will make this clear.

Table III

| | Inorganic P | | |
|---|---|---|---|
| | I | II | III |
| Sodium hexosediphosphate | 0·70 | 0·75 | 0·77 |
| Sodium phosphoglycerate | 0·41 | 0·56 | 0·64 |

All coenzymes are present in both cases.

Here the hexosediphosphate gives more free inorganic P than an equivalent amount of phosphoglycerate. This can only mean that some other dephosphorylating mechanism is at work for hexosediphosphate, besides that acting after its breakdown to phosphoglycerate. The same would seem to be true of an ordinary muscle extract as the following experiment with an extract kept for several days to destroy adenylpyrophosphatase shows:

| Substrate | Lactic acid | Inorganic P |
|---|---|---|
| Hexosediphosphate | 0·63 | 0·42 |

The inorganic P is almost double the amount corresponding to the lactic acid. In drawing conclusions from the appearance or disappearance of inorganic P in experiments where hexosediphosphate is undergoing oxido-reduction this possibility must always be kept in mind.

*Dephosphorylation of sodium phosphoglycerate*

As has been mentioned before, the acetone powder extracts used for these experiments do not contain any appreciable amounts of adenylpyrophosphatase but have a strong dephosphorylating action upon phosphoglyceric acid. The extracts were frequently tested during the course of this work but in no

circumstances was any dephosphorylating action upon adenylpyrophosphate found. The following figures show the results of a typical experiment:

| | Inorganic P | |
|---|---|---|
| Adenyltriphosphate (0·28 mg. pyro-P) | 0·02 | Extract dialysed 3 hr. |
| Sodium phosphoglycerate (1·5 mg. P) | 0·56 | |

The extract itself produced no free inorganic P under these conditions.

The requirements of coenzymes for this dephosphorylation were now investigated. As, however, the enzyme catalysing this reaction fell off in activity by long dialysis the extract could not be completely freed from the coenzymes already present by this method.

*Effect of dialysis on the dephosphorylation of phosphoglycerate*

| | Inorganic P | | |
|---|---|---|---|
| Extract dialysed for ... | 3 hr. | 22 hr. | 88 hr. |
| | 0·56 | 0·39 | 0·19 |
| | — | — | 0·17 |

However, the results in the following table are fairly clear and show that both magnesium and adenylpyrophosphate are necessary. It is not possible to make any conclusions about coenzyme I as it is well known that it is difficult to dialyse away the last traces of this and the requirement for it is very small, but that it can replace A.T.P. to some extent as a phosphate transporter seems clear enough.

Table IV

| | Inorganic P | |
|---|---|---|
| Extract dialysed 22 hr. | | |
| No coenzymes | 0·10 | 0·12 |
| Mg | 0·16 | 0·16 |
| Mg + A.T.P. | 0·38 | 0·34 |
| Mg + A.T.P. + coenzyme I | 0·39 | 0·33 |
| Mg + coenzyme I | 0·27 | — |
| A.T.P. + coenzyme I | 0·26 | 0·26 |

The optimum concentrations of coenzymes and substrate are similar to those in normal glycolysis. With 0·025 *M* sodium phosphoglycerate and 0·0006 *M* A.T.P. and 0·003 *M* Mg 50 % of the maximal values are reached. The velocity increases with increasing concentrations of phosphoglycerate up to 0·07 *M* and at this high concentration of phosphoglycerate slightly with increased A.T.P. and magnesium.

For identifying the end product of the dephosphorylation the total bisulphite-binding substances formed were estimated according to the method of Clift & Cook [1932]. Reckoning these as pyruvic acid an amount roughly equivalent to the inorganic phosphate set free was found after making due correction for any phosphopyruvic acid which might be present.

| Total pyruvic acid | Present as phosphopyruvic acid | Free pyruvic acid | Equivalent P that should be set free | P found |
|---|---|---|---|---|
| 1·69 | 0·38 | 1·31 | 0·46 | 0·35 |

As under these conditions sodium glycerate added to muscle extract does not give any bisulphite-binding substance it may be concluded that the dephosphorylation takes place after conversion into phosphopyruvic acid. This is moreover confirmed by the fact that the dephosphorylation is inhibited by fluoride as usual. *M*/200 iodoacetate does not inhibit the reaction.

In trying to find out the mechanism of this dephosphorylation, in which apparently adenylpyrophosphate plays a prominent part, various possibilities were tested. Since hexosediphosphate is able to decompose in such an extract into hexosemonophosphate and inorganic phosphate and adenylpyrophosphate can rephosphorylate the hexosemonophosphate it is easy to conceive of a small trace of hexosediphosphate, acting catalytically, converting the phosphate from adenylpyrophosphate into inorganic form, the resulting adenylic acid being immediately rephosphorylated by the phosphopyruvic acid. The necessity for both A.T.P. and magnesium would be explained by this as also the dispensability of coenzyme I. It is practically impossible to make sure that a trace of hexosediphosphate or hexosemonophosphate does not occur in the phosphoglycerate especially in view of the nature of its preparation from hexosediphosphate. However, one would expect in such a case that a trace of hexosediphosphate added to an extract would catalyse the breakdown of adenylpyrophosphate. Experiments were done in which small amounts of hexosediphosphate (0·1 mg. P) were added to an extract acting upon fairly large amounts of adenylpyrophosphate (0·55 mg. pyro-P) but no activation of the dephosphorylation could be obtained over and above that due to the added hexosediphosphate itself. Similar amounts of hexosediphosphate also failed to produce any activation of the dephosphorylation of phosphoglycerate when added to an extract acting upon that substrate.

There is however some ground for believing that when large quantities of hexosediphosphate are present adenylpyrophosphate can be dephosphorylated in this way. Thus when an extract acts upon fairly equivalent amounts of hexosediphosphate and phosphoglycerate at the same time the amount of inorganic P produced is much less than the sum of the individual dephosphorylations when they are acted upon separately. While this may be due partly to some of the triosephosphate being removed by dismutation with the pyruvic acid formed as a result of the dephosphorylation of phosphoglycerate (this reaction being much faster than the oxido-reduction of triosephosphate and subsequent dephosphorylation), thus upsetting the equilibrium hexosediphosphate→triosephosphate, the difference as will be evident from the table is too great to be accounted for in this way. It is therefore legitimate to conclude that some of the phosphorus from phosphopyruvic acid is being conveyed by adenylic acid to rephosphorylate hexosemonophosphate. The effect of the coupled esterification of adenylic acid consequent upon the dismutation is unlikely to be prominent, due to the lack of free adenylic acid.

Table V

| | Inorganic P | |
|---|---|---|
| Hexosediphosphate only | 1·02 | 0·39 |
| Phosphoglycerate only | 0·57 | 0·36 |
| Hexosediphosphate + phosphoglycerate | 1·23 | 0·40 |

Another possibility that was considered [*v.* Ohlmeyer & Ochoa, 1937] is that reduced coenzyme I might act as a phosphate acceptor from adenylpyrophosphate, being then itself broken down by a cozymase pyrophosphatase. Traces of hexosediphosphate present might then be able to reduce the coenzyme, enabling it to act in this way. Addition of traces of hexosediphosphate to an extract acting upon adenylpyrophosphate in the presence of coenzyme I failed however to produce any extra dephosphorylation beyond that of the added hexosediphosphate. The dephosphorylation of phosphoglycerate was also not increased by the addition of both coenzyme I and traces of hexosediphosphate.

Moreover, the fact that iodoacetate does not inhibit the reaction makes this possibility even more unlikely.

The influence of other substances like sodium pyruvate, sodium glycerate and large amounts of coenzyme I on the dephosphorylation of adenylpyrophosphate was tested but none of them gave any increase of inorganic P. Finally, small amounts of phosphoglycerate (0·1 mg. P) were also found not to have any activating effect on the breakdown of adenylpyrophosphate.

Since phosphoglycerate is so readily dephosphorylated by these extracts it seemed to be of great interest to find out whether creatinephosphate could be dephosphorylated under these conditions. As creatinephosphate decomposes more readily at a lower *p*H than that at which most of the other experiments were carried out (about 8·6) the solutions were brought to about *p*H 7·4 by bubbling a gas mixture of 5 % $CO_2$ and 95 % nitrogen through them and keeping them tightly stoppered during the course of the experiment. Both inorganic P and creatinephosphate P were estimated. No breakdown of creatinephosphate was observed.

The effect of adding a phosphate acceptor such as creatine during the dephosphorylation of phosphoglycerate was tried and showed very large amounts of creatinephosphate accumulating. Very little inorganic P was formed.

| | Inorganic P | Creatine-phosphate P |
|---|---|---|
| Phosphoglycerate | 0·35 | — |
| + creatine (13 mg.) | 0·14 | 0·95 |

Finally it remained to test how much inorganic P would be formed from phosphoglycerate by a fresh extract containing adenylpyrophosphatase.

| | Inorganic P |
|---|---|
| (1) Adenylpyrophosphate (0·60 mg. pyro-P) | 0·29 |
| (2) A.T.P. (0·09 mg. pyro-P) + sodium phosphoglycerate (1·5 mg. P) | 0·60 |

Here, even though the extract has produced only 0·29 mg. P from a large excess of adenylpyrophosphate, it has produced 0·60 mg. P from sodium phosphoglycerate with only a seventh of the amount of adenylpyrophosphate to act as carrier, showing that even in a fresh extract there must be mechanisms producing dephosphorylation of phosphoglycerate other than through adenylic acid formed by dephosphorylation of A.T.P. by adenylpyrophosphatase.

In conclusion one might refer to the velocity of the dephosphorylation of phosphoglycerate in an acetone powder extract. It is interesting because of the very steady rate at which it proceeds as will be evident from the following table:

Table VI

| | Inorganic P produced |
|---|---|
| 10 min. at 38° | 0·19 |
| 20 ,, | 0·29 |
| 30 ,, | 0·36 |
| 60 ,, | 0·50 |

The action of arsenate upon this dephosphorylation will now be considered.

*The action of arsenate*

Arsenate increases the rate of dephosphorylation of phosphoglycerate enormously even in very small concentrations, a fact which has been observed before [Meyerhof & Kiessling, 1935].

Table VII

| | Inorganic P | |
|---|---|---|
| No arsenate | 0·09 | Extract dialysed 36 hr. A.T.P. and Mg |
| 0·005 *M* arsenate | 0·92 | added. Time of exp. 15 min. at room |
| 0·0025 *M* arsenate | 0·77 | temperature |
| 0·002 *M* arsenate | 0·69 | |
| 0·001 *M* arsenate | 0·57 | |
| 0·0002 *M* arsenate | 0·36 | |
| 0·0001 *M* arsenate | 0·30 | |

With *M*/10,000 arsenate the rate is still at least three times the rate in the absence of arsenate while with *M*/200 arsenate it is as much as ten times. That the dephosphorylation proceeds normally with production of pyruvate was shown by estimation of the pyruvic acid formed according to the method of Clift & Cook. The next point is to find what coenzymes if any are required for this reaction with arsenate. This is easier than with the dephosphorylation in the absence of arsenate since it was found that the activation by arsenate was not damaged seriously by very long dialysis.

| | Inorganic P | |
|---|---|---|
| Extract dialysed 22 hr. | 1·22 | 15 min. at room temp. Mg, A.T.P. |
| 88 hr. | 0·97 | and cozymase added |

As the following table shows, 79 hr. dialysis is sufficient to remove most of the coenzymes and the necessity of all the three coenzymes, A.T.P., cozymase and magnesium for this activation by arsenate is quite clear.

Table VIII

| | Inorganic P | |
|---|---|---|
| No addition | 0·03 | |
| Mg only | 0·14 | |
| Mg + A.T.P. | 0·34 | 30 min. at room temp. |
| Mg + adenylic acid (0·3 mg.) | 0·16 | Arsenate *M*/1000 |
| Mg + coenzyme I | 0·11 | |
| Mg + A.T.P. + coenzyme I | 1·04 | |
| Mg + adenylic acid + coenzyme I | 1·25 | |
| Coenzyme I + adenylic acid | 0·04 | |

The adenylpyrophosphate was found to contain traces of cozymase; adenylic acid was therefore used as a check.

It now remains to elucidate the mechanism of this reaction. Experiments showed that adenylpyrophosphate is not broken down to adenylic acid and inorganic phosphate in the presence of arsenate, coenzyme I and magnesium. Reduced coenzyme I prepared by reducing coenzyme I with sodium hydrosulphite according to Euler & Hellström [1938], small amounts of hexosediphosphate and pyruvate (0·0003 and 0·0006 *M* respectively) separately and together were all found not to activate the dephosphorylation of adenylpyrophosphate in the presence of arsenate. A sample of phosphoglycerate prepared after treating it with *N* HCl at 100° for 4 hr. to remove any easily hydrolysable phosphate compounds including hexosediphosphate was found to produce inorganic phosphate in the presence of arsenate as rapidly as the original phosphoglycerate itself. One must of course remember that if any hexosemonophosphate was present it was not likely to decompose completely under these conditions.

The effects of the two poisons, fluoride and iodoacetate, were next tried and both were found to inhibit the activation by arsenate.

Table IX

| | Inorganic P | | |
|---|---|---|---|
| Arsenate only $M/400$ | 0·99 | 1·23 | 30 min. at room temp. |
| + fluoride $M/25$ | 0·08 | — | |
| + iodoacetate $M/840$ | — | 0·20 | |

It is interesting to notice that, using a yeast enzyme preparation in the presence of arsenate, Meyerhof *et al.* [1937] found that phosphopyruvic acid is readily broken down to pyruvic acid and free phosphate, provided that cozymase, a trace of hexosediphosphate and an active oxido-reduction enzyme system are present. This mechanism of dephosphorylation has not so far been explained. It also is inhibited by iodoacetate.

The action of iodoacetate is dissimilar from its effect on the dephosphorylation of phosphoglycerate in the absence of arsenate where it does not inhibit the reaction. In order to make sure that the arsenate and iodoacetate were not for some reason neutralizing each other's effects the following experiment was tried, making use of the fact that iodoacetate inhibits the dismutation between triosephosphate and pyruvic acid. If now the effects of arsenate and iodoacetate were to antagonize each other arsenate should remove the inhibition of this dismutation by iodoacetate and we should get the same amount of lactic acid formed.

Table X

| | Lactic acid |
|---|---|
| Triosephosphate + pyruvate only | 2·4 |
| + iodoacetate | 0·3 |
| + arsenate | 4·5 |
| + arsenate + iodoacetate | 0·3 |

Time of exp. 30 min. at 38°

The inhibition by iodoacetate therefore seems to be quite specific and not influenced by arsenate.

It has been mentioned before that small amounts of pyruvate had no action upon adenylpyrophosphate in the presence of arsenate and the coenzymes. With much larger amounts of pyruvate (0·004–0·020 $M$) a small amount of dephosphorylation can be observed. This dephosphorylation is also inhibited by iodoacetate.

Table XI

| | Inorganic P | |
|---|---|---|
| Extract dialysed 88 hr. | | |
| A.T.P. (0·3 mg. pyro-P) only | 0·01 | 30 min. at 38° |
| A.T.P. + 0·004 $M$ pyruvate | 0·05 | |
| A.T.P. + 0·020 $M$ pyruvate | 0·10 | Arsenate $M/1000$ |
| A.T.P. + 0·020 $M$ pyruvate + iodoacetate $M/800$ | 0·01 | |

The reason for this dephosphorylation is not known. It may be due to certain impurities in the pyruvate but the amount is perhaps too small to be of significance.

Finally the effect of arsenate on creatinephosphate breakdown in the presence and absence of coenzymes was studied.

The results (Table XII) show that creatinephosphate is to some extent broken down when arsenate is present although quite stable in its absence. There are, however, some great differences between the breakdown of creatinephosphate and that of phosphoglycerate. The former as will be evident from the table is hardly

Table XII

| Arsenate $M/1250$ present in all | Inorganic P |
|---|---|
| Creatinephosphate only (0·98 mg. P) | 0·15 |
| + Mg + A.T.P. + cozymase | 0·17 |
| + Mg + A.T.P. + cozymase + pyruvate (0·02 $M$) | 0·28 |
| + Mg + A.T.P. + cozymase + hexosediphosphate ($M/4000$) | 0·29 |
| + Mg + A.T.P. + cozymase + iodoacetate $M/800$ | 0·04 |
| Phosphoglycerate only | 0·20 |
| Time of exp. 30 min. at 38° | |
| Extract dialysed 88 hr. | |

affected by the absence of the coenzymes, which produce no appreciable increase in inorganic phosphate. Pyruvate produces a small increase comparable with that which it produces with A.T.P. (see Table XI) and hexosediphosphate in like amount. Iodoacetate inhibits the reaction at a concentration of $M/800$ though at this concentration it hardly affects the transfer of phosphate from creatinephosphate to adenylic acid. It may also be noted that phosphoglycerate under the same conditions (in the absence of all coenzymes) gives a similar amount of inorganic phosphate to that given by creatinephosphate in this experiment but with all coenzymes it gives as much as 3·32 mg. P or about 16–20 times the amount.

## Discussion

It is abundantly clear from these experiments that dephosphorylation of phosphoglycerate in muscle extracts involves several different mechanisms all of which seem to require one thing in common, adenylic acid. When glycogen is present to act as a phosphate acceptor the adenylic acid is easily regenerated and then re-esterified. The action of adenylpyrophosphatase is also clear. It is when both these are lacking that the two mechanisms of dephosphorylation treated here become more prominent and clear.

That the phosphoglycerate passes through the stage of phosphopyruvate and then breaks down into pyruvate and phosphate is clear both from the estimations of pyruvate formed as well as from the fact that the dephosphorylation both in the absence as well as in the presence of arsenate is poisoned by fluoride. We shall now consider briefly in review the evidence for the nature of the dephosphorylation first in the absence of arsenate, and secondly when arsenate is present.

*Dephosphorylation without arsenate.* It is clear from the figures given before that this dephosphorylation requires both Mg and adenylpyrophosphate for full activation. Addition of cozymase does not seem to increase the rate of breakdown. This may be due to there being already a sufficiency of this coenzyme in the extract as it is known to be required only in minute quantities and it is difficult to dialyse the last traces away completely. However, it will be seen that it can replace adenylpyrophosphate to some extent but is not quite so active.

The absence of adenylpyrophosphatase from the extracts has been demonstrated. Large quantities of adenylpyrophosphate added to the extract do not give any measurable increase in inorganic phosphate even when cozymase and magnesium are added. It remains therefore to see how the adenylpyrophosphate can serve as phosphate acceptor for the phosphate of phosphoglycerate, assuming that it is the only way in which it can catalyse the dephosphorylation.

Two possibilities have been considered.

(1) Setting free of phosphate by the system adenylpyrophosphate, hexosemonophosphate, hexosediphosphate and hexosediphosphatase

$$\text{Hexosediphosphate} \xrightarrow{\text{Phosphatase}} \text{Hexosemonophosphate} + \text{inorganic P}$$

$$\text{Hexosemonophosphate} + \text{adenylpyrophosphate} \rightarrow \text{hexosediphosphate} + \text{adenylic acid}$$

$$\text{Adenylic acid} + \text{phosphopyruvate} \rightarrow \text{pyruvate} + \text{A.T.P.}$$

(2) Setting free of phosphate by the system adenylpyrophosphate, reduced cozymase, reduced cozymase pyrophosphate and reduced cozymase pyrophosphatase with traces of hexosediphosphate serving to form an oxido-reduction system for reducing cozymase in the first instance.

$$\text{Reduced cozymase pyrophosphate} \xrightarrow[\text{reduced cozymase pyrophosphatase}]{} \text{Reduced cozymase} + \text{inorganic P}$$

$$\text{Reduced cozymase} + \text{A.T.P.} \longrightarrow \text{adenylic acid} + \text{reduced coenzyme pyrophosphate}$$

$$\text{Adenylic acid} + \text{phosphopyruvate} \longrightarrow \text{A.T.P.} + \text{pyruvate}$$

Since traces of hexosediphosphate which might be supposed to reduce cozymase do not activate the dephosphorylation of either A.T.P. or phosphoglycerate with or without cozymase, and the dephosphorylation of phosphoglycerate, moreover, is not inhibited by iodoacetate, it must be considered that no evidence exists for either of these mechanisms.

Finally the idea that the dephosphorylation is the result of the action of a phosphopyruvic phosphatase might be considered but for the fact that it is hard to imagine a phosphatase so specific as to be activated by adenylpyrophosphatase or cozymase.

*Dephosphorylation in the presence of arsenate.* Here also the phosphoglycerate passes through the phosphopyruvic stage before dephosphorylation, as shown by the formation of pyruvate and by the inhibition of the reaction by fluoride. The reaction needs all the three coenzymes, adenylpyrophosphate, magnesium and cozymase for full activation. The poisoning of the activity by iodoacetate might lead one to think that here indeed the coenzyme I is taking part in a system of oxido-reduction with perhaps traces of hexosediphosphate. However, in this case also, as before, small amounts of added hexosediphosphate alone and along with pyruvate were not found to give any increase of dephosphorylation of either adenylpyrophosphate or phosphoglycerate or of creatinephosphate in the presence of all the coenzymes. Reduced cozymase added to A.T.P. also does not have any noticeable action. One must wait for further experimental evidence before any idea of the true role of cozymase in the mechanism can be formulated.

The stimulation of the breakdown of creatinephosphate in the presence of arsenate and its poisoning by iodoacetate would tend to show that it is perhaps of similar character. Against this, however, is the fact that addition of coenzyme does not activate this breakdown and that the activity itself is very slight compared with the dephosphorylation of phosphoglycerate under the same conditions. The slight activation of adenylpyrophosphate breakdown by pyruvate is perhaps also of similar origin.

A word might be added about arsenate activation in general in these extracts. It was found that while the arsenate did not activate the production of lactic acid from glycogen and hexosediphosphate both the dismutation between triose-

phosphate + pyruvate as well as the dephosphorylation of phosphoglycerate are considerably accelerated showing that arsenate perhaps acts only in the later stages of glycolysis in such an extract.


## Summary

1. Of the three phosphatases present in fresh muscle extract, inorganic pyrophosphatase, adenylpyrophosphatase and hexosediphosphatase which breaks down hexosediphosphate into hexosemonophosphate and inorganic P, the adenylpyrophosphatase is the least stable, but it is not completely destroyed even after 2 weeks at 0° and 16 hr. dialysis. Attempts to separate the two pyrophosphatases by adsorption did not meet with success though there was some evidence that they were distinct and separate enzymes.

2. An acetone powder extract of muscle is quite free from adenylpyrophosphatase even though the other two phosphatases are present in active form. Such an extract does not dephosphorylate adenylpyrophosphate but readily dephosphorylates phosphoglycerate when small amounts of magnesium and adenylpyrophosphate are added. Creatinephosphate is not broken down under these conditions. The dephosphorylation is inhibited by fluoride but not by iodoacetate.

3. The dephosphorylation of phosphoglycerate is activated greatly by arsenate. A.T.P., magnesium and cozymase are necessary for this activation. Fluoride and iodoacetate inhibit it. Creatinephosphate is slightly broken down in the presence of arsenate.

4. Two possibilities were considered to explain the mechanism of the reaction by which A.T.P. could serve as phosphate acceptor in the absence of any known mechanism for its breakdown into adenylic acid. One assumed the presence of traces of hexosemonophosphate formed by dephosphorylation of hexosediphosphate by the phosphatase present, whilst the other postulated the presence of reduced cozymase acting as phosphate carrier. So far no experimental evidence for either of these mechanisms has been obtained.


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